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Factors Affecting Root Colonization by Calonectria Ilicicola and Development of Red Crown Rot Disease on Soybean.

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FACTORS AFFECTING ROOT COLONIZATION BY *CALONECTRIA ILICICOLA* AND DEVELOPMENT OF RED CROWN ROT DISEASE ON SOYBEAN

A Dissertation

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in

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To my loving parents

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ABSTRACT

Red crown rot of soybean caused by *Calonectria ilicicola* is a serious disease in Louisiana. The pathogen infects soybean roots and the above ground symptoms and signs appear during reproductive stages of the plant. Little information is available on root infection as well as effects of environmental factors or varietal resistance on this process. Field studies were conducted in 1994, 1995, and 1996 to determine the effects of planting date, cultivar susceptibility, and soil pathogen population on soybean root colonization by *C. ilicicola* and subsequent disease development. Early season colonization of tap as well as lateral roots was important for red crown rot symptom development. Symptom development in more susceptible Sharkey was reduced following delayed planting but remained low in less susceptible Cajun regardless of planting date. Root colonization correlated positively with soil pathogen levels. A weak positive correlation was detected between taproot colonization and pathogen population level during all 3 growing seasons. In the case of lateral root colonization, a strong positive correlation was detected in 1994, the only year that foliar disease symptoms were detected. Pathogen population changes in the experimental field were not consistent during 3 growing seasons. Considerable decrease in the pathogen population in soil in 1995, along with reduced soybean root colonization could be attributed to high soil temperature experienced during that summer.

High temperature effects on *C. ilicicola* microsclerotia in heavy alluvial soil were examined. Microsclerotia survived at soil temperatures between 20-35^{0}C. Optimal infectivity of microsclerotia was detected,

when microsclerotia were incubated in soil between 25-30^{0}C. Results supported the role for high soil temperature in controlling field pathogen population in 1995. Temperature effects on soybean root colonization by *C. ilicicola* were examined in growth chambers. The optimal soil temperature range for root colonization was between 20-30^{0}C.

The effect of plant age on soybean root colonization by *C. ilicicola* was examined by exposing the pathogen to plants at different ages. Soybean plants were most susceptible to *C. ilicicola* during the first week after seedling emergence. Susceptibility was then reduced to nearly half and fluctuated at that level till the end of 8 weeks.

CHAPTER 1

INTRODUCTION

Soybean, *Glycine* max (L.) Merrill belongs to the family Leguminosae, subfamily Papilionoideae, and tribe Phaseoleae. It is one of the oldest cultivated crops known to mankind and is believed to have originated in Asia, as ancestors of this cultivated species are found in China and Korea (Chapman and Carter, 1976).

Soybean was introduced to the United States in the mid 1760's (Hymowitz and Harlan, 1983). In the 19th century, occasional references to soybean were made in scientific literature. Soybean research intensified after 1890, and 2 bulletins solely on soybean were published by USDA between 1899 and 1900 (Probst and Judd, 1973). In the United States, soybean initially was a forage crop, but rapidly became an important oil seed crop (Chapman and Carter, 1976). Soybean now accounts for about 82% of the nation's oils and fats (Anonymous, 1997) and also is the choice of protein for the livestock and poultry industry (Smith, 1994).

Today the United States is one of the four major producers of soybean in the world and is responsible for one-half of the total world soybean production [Morrison and McCormick, 1996). Brazil, Argentina, and China are the other major soybean producers [Morrison and McCormick, 1996). Presently soybean is grown in 29 states in the western corn belt, eastern corn belt, southeastern, delta, and Atlantic states. In 1997, 64.8 million metric tons (2,382 billion bushels) of soybeans were produced in the United States with the value of nearly $16,317 million (Anonymous, 1997). Soybean production in Louisiana peaked in 1979 with 2.55 million metric tons (93.8 million bushels) produced on 1.36 million hectares (3.35 million acres), which was valued at $600 million (Morrison and McCormick, 1996). Although it is not as important as it

once was, soybean is still one of the five major crops in Louisiana, with a value of nearly $ 268 million in 1997. Recent estimates showed 14.2% yield loss due to diseases in Louisiana (Pratt, 1998).

Soybean is a warm-season, herbaceous annual, and photoperiod is the important factor determining the area of adaption of this crop (Hartwig, 1973). Depending on response to day length, 12 maturity groups (00 through X) have been established to identify the region of adaptation for soybean in North America. Group 00 cultivars bloom and develop seeds during longer days in northern areas whereas group X cultivars mature in tropical latitudes (Hartwig, 1973). A stage of development system was adapted to accurately identify the plant developmental stages independent of date or latitude. The preferred index in this respect is based on Fehr *et al.* (1971), who designated soybean growth stage by letter (V = vegetative or R = reproductive) followed by a number (Teare and Hodges, 1994). The number after "V" indicates the number of nodes, whereas the number after "R" indicates the state of bloom, pod, or seed maturity.

The optimum planting date for soybean varies from mid-April to late June, depending on variety and location in North America. Initiation of soybean planting in the nothern United State is determined mostly by temperature. Minimum temperature for rapid germination and emergence is 15.5^{0}C (Boquet, 1994). In the southern United States, soybean planting time is determined mostly by daylength. Determinate cultivars grown in the south require a critical daylength of about 14.5 hrs to produce maximum potential yield of that cultivar. As this daylength is not reached until mid-may in the south, the optimum soybean planting time is

mid-May in the southern states. In most regions of the United States, soybean yield is reduced 20 to 27 kg per hectare for each day of planting after June 20 (Boquet, 1994).

Red crown rot, caused by the fungus *Calonectria ilicicola* Boedijn and Reitsma, is an important disease of soybean (Rowe *et al.*, 1973; Berggren and Snow, 1989). In 1966, Bell and Sobers described this fungus as the causative agent of pod, peg, and root necrosis of peanut (*Arachis hypogea*) and named it *Calonectria crotalariae* (Bell and Sobers, 1966). In 1993, Crouse *et al.* considered morphological similarities as well as protein banding patterns of the type culture of *C. ilicicola* Boedijn and Reitsma isolated from potatoes in Java and the isolate of *C. crotalariae*, and reported that these 2 names had been given to the same fungus (Crouse *et al.*, 1993). Since *C. ilicicola* Boedijn and Reitsma was described before *C. crotalariae* Bell and Sobers, *C. ilicicola* Boedijn and Reitsma became the accepted name for this fungus, with *Calonectria theae* Loos var. *ilicicola* Loos and *C. crotalariae* (Loos) Bell and Sobers as synonyms (Crouse *et al.*, 1993). The name *Cylindrocladium parasiticum* Crouse was justified for the anamorph of this fungus described by Bell and Sobers as *Cylindrocladium crotalariae* (Crouse *et al.*, 1993).

Calonectria ilicicola belongs to the order Hypocreales, class Pyrenomycetes in the phylum Ascomycota. This fungus produces reddish brown perithicia. Club-shaped asci with 8 curved, hyaline 1-(3) septate ascospores, are produced in these perithecia. This fungus produces 3 septate, hyaline conidia that are straight and cylindrical (Berggren and Snow, 1989). The roles of conidia and ascospores in the disease cycle are not known. Irregularly shaped microsclerotia (52 x 74 μm - 70 x 103 μm) (Rowe *et al.*, 1974; Diomande and Beute, 1981) are the survival and

dispersal structures produced of this fungus that can survive several years in soil or on host debris (Bell and Sobers, 1966). Jonston and Beute (1975) reported that microsclerotia were formed in the cortex of infected peanut roots 56-84 days after inoculation. Microsclerotia are spread in soil by movement of root fragments (Rowe *et al.*, 1973; Rowe *et al.*, 1974) and with run-off or flood water (Berner *et al.*, 1986).

Calonectria ilicicola has a broad host range. In addition to red crown rot in soybean (Rowe *et al*, 1973), this fungus causes black root rot (also known as Cylindrocladium black rot) of peanut (*Arachis hypogea*) (Bell and Sobers, 1966). It also was reported to cause disease on koa (*Acacia koa)* (Aragaki *et al.* 1972), papaya (*Carica papaya*) (Nishijima and Aragaki, 1973), blueberry (*Vaccinium corymbosum*) (Miholland, 1974), leea (*Leea cocinema*) (Ko *et al.*, 1981), palm (*Howeia forterana*) (Uchida and Aragaki, 1992), and has been found consistently pathogenic on indigo (*Indigofera tinctoria*) (Berner *et al.*, 1988).

Calonectria ilicicola on soybean was first reported in Louisiana in St. John the Baptist Parish in 1976 (Berner *et al.*, 1986). Red crown rot was reported in 17 parishes along the Mississippi River 9 years later and was considered an important fungal disease in Louisiana (Berner *et al.*, 1988). Berner *et al.* (1988) suggested that the movement of *C. ilicicola* from former indigo plantations by flood water accounted for the distribution of the pathogen in Louisiana.

Foliar symptoms of red crown rot usually appear during beginning pod (R_3) to full pod (R_4) soybean growth stages and include leaf chlorosis and interveinal necrosis followed by defoliation (Berggren and Snow, 1989).

The fungus colonizes roots, and reddish-brown perithecia appear in the crown region of the plant beginning at R_3 to R_4. Perithecia are diagnostic signs of the disease (Berggren and Snow, 1989). *Calonectria ilicicola* causes root discoloration and necrosis in peanut roots (Bell and Sobers, 1966), as the name 'black root rot' implies. Tomimatsu and Griffin (1982) observed numerous infections (1 to >1,000 per plant) 21 days after planting on asymptomatic tap, lateral, and fine peanut roots grown in soils naturally infested with *C. ilicicola*. Root colonization by *C. ilicicola* apparently occurs well ahead of necrotic lesion appearance in peanut roots (Tomimatsu and Griffin, 1982). Our observations revealed that soybean root infection by this fungus does not necessarily cause root discoloration (unpublished data).

Black root rot on peanut has been studied in much greater detail than has red crown rot of soybean. Development of black root rot on peanut is reported to be affected by host susceptibility (Krigsvold *et al.*, 1982; Black *et al.*, 1984) as well as biotic factors (Black and Beute, 1985) including nematodes (Diomande and Beute, 1981; Culbreath *et al.*, 1992) and abiotic factors such as soil nutrients, temperature, and moisture (Black *et al.*, 1984; Phipps and Beute, 1979; Pataky and Beute, 1983; Pataky *et al.*, 1984). Germination of microsclerotia of *C. parasiticum* (previously known as *C. crotalariae*), the anamorph of *C. ilicicola*, was significantly higher in the rhizosphere of susceptible than resistant cultivars (Krigsvold *et al.*, 1982). Diomande and Beute (1981) reported a positive correlation between *Meloidogyne hapla* and *C. ilicicola* populations in field soils. Overstreet *et al.* (1990) discussed interactions between *C. ilicicola* and *Heterodera glycines* on

soybean. Increases in densities of actinomycetes and bacteria and copper levels in peanut and soybean field soils infested with microsclerotia of *C. ilicicola* was associated with decreases in root rot severity on peanut (Black and Beute, 1985). Nitrogen fertilizers also appear to reduce the incidence of black root rot incidence in peanut (Pataky *et al.*, 1984). Phipps and Beute (1979) suggested that winter temperatures are a primary determinant of the longevity of microsclerotia of *C. ilicicola* in North Carolina fields. They also believed that the absence of adequate soil moisture during the 1975 growing season accounted for the absence of black root rot of peanut in their microplot experiments (Phipps and Beute, 1979). Pataky and Beute (1983) suggested that soil temperature and moisture interact in their effects on the viability of microsclerotia.

Soil temperature is reported to be one of the most critical environmental factors affecting development of black root rot in peanut (Bell, 1967; Phipps and Beute, 1977). Black *et al.* (1984) observed that disease severity in microplots was greater when peanuts were planted on 2 May (minimum soil temperature $<18^0$C) than on 17 or 30 May (minimum soil temperature $\geq 18^0$C). Root necrosis increased as soil temperature increased from 15^0C to 25^0C, but did not change significantly thereafter up to 40^0C (Bell, 1967). Phipps and Beute (1977) reported that, under greenhouse conditions, a soil temperature of 25^0C and moisture content near field capacity were most conducive for infection and rot of peanut roots by *C. ilicicola*. Contrary to the report of Bell (1967), Phipps and Beute (1977) observed root colonization, and rot increased with increasing soil temperature from 20^0C to 25^0C, but decreased thereafter. No measurable

root colonization and rot were observed at 35°C. Phipps and Beute (1977) also reported that neither root colonization nor plant growth reduction was observed in soils exposed to 35°C for 9 hours during daylight and then to a temperature of 25°C for the remainder of the day. In their opinion, this indicated that the duration of diurnal exposure to higher temperatures may be critical in the development of the disease. Phipps and Beute (1977) also observed higher root rot severity in infested wet soil.

Temperature affects the host as well as the pathogen. Selecting a planting time which is best for the host growth, but that reduces pathogen activities, is a cultural tactic used in disease management. Delayed planting may be associated with increased soil temperature that reduces soilborne plant pathogen activities, such as germination and pathogenic aggressiveness or survival of overwintering structures. Late planting is one of the cultural practices used in disease management of black root rot in peanut (Sidebottom and Beute, 1989). It also was reported that late planting reduces the incidence of red crown rot in soybean (Russin *et al.*, 1985; Berner *et al.*, 1988). However, delayed planting also shortens the vegetative growth period of the soybean plant, which may in turn reduce yield. Therefore, it is important to know the best time to plant soybean to minimize disease loss and to minimize yield loss due to late planting. Less susceptible soybean cultivars are also used in red crown rot management. Delaying planting combined with lower cultivar susceptibility may be useful for management of red crown rot in soybean.

Although complete host resistance has not been demonstrated, some soybean cultivars are known to be less susceptible to red crown rot.

However, no attempts have been made to understand the genetic basis of soybean host resistance to *C. ilicicola*. Host resistance is used to manage black root rot in peanut even though resistant peanut cultivars are not immune to *C. ilicicola*. Pathogen entry into the peanut vascular system through the taproot is prevented by production of periderm in less susceptible cultivars (Harris and Beute, 1982). Disease severity in susceptible and resistant cultivars can depend on microsclerotia densities in soil (Harris and Beute, 1982). Reduction of inoculum density therefore is important in limiting disease severity in resistant cultivars. Reduction of initial inoculum densities through cultural and chemical methods such as crop rotation and soil fumigation (Phipps, 1990) are recommended for black root rot management in peanut (Sidebottom and Beute, 1989). Therefore, factors affecting the survival of fungal microsclerotia in the field may be of critical importance. Available data suggest that effective inoculum density in the field is fully or partially controlled by temperature and soil moisture (Phipps and Beute, 1977; Griffin *et al.*, 1978). Seasonal as well as diurnal fluctuations in these two factors might influence the level and/or viability of survival propagules of the pathogen, which in turn may affect the incidence and severity of disease.

The effect of low temperature on survival and germinibility of microsclerotia of *C. ilicicola* in peanut soils was studied by several workers (Griffin *et al.*, 1978; Roth *et al.*, 1979; Taylor *et al.*, 1981; Pataky and Beute, 1983). Fewer microsclerotia were recovered from naturally infested soil when incubated at -3^{0}C than at 5^{0}C, and no germinable microsclerotia were found in soils incubated at -10^{0}C even for 1 week (Roth *et al.*, 1979).

When soils incubated at -10^{0}C or at 6^{0}C for 4 weeks were transferred to 26^{0}C for 4 weeks, the low temperature effect was partially alleviated, indicating that low temperature does not always cause a permanent loss of viability in microsclerotia (Roth *et al.*, 1979). Griffin *et al.* (1978) reported that microsclerotia were not recovered from soil incubated at 6^{0}C for 1 month, but incubation at 26^{0}C had little or no effect on recovery of microsclerotia. Germinability of microsclerotia decreased progressively over 4 weeks when naturally infested soils were incubated at 6^{0}C (Roth *et al.*, 1979). Phipps and Beute (1979) and Taylor *et al.* (1981) reported that extreme winter cold was associated with a considerable decrease in germinable microsclerotia.

The effects of high temperature on the survival of *C. ilicicola* microsclerotia and infectivity of microsclerotia after exposure to high soil temperature has had little investigation. However, high temperature effects on the survival structures of several other soilborne pathogens have been studied in some detail. Bega and Smith (1962) and Sheikh and Ghaffar (1986) discussed high temperature effects on the survival and time-temperature relationships for the thermal inactivation of microsclerotia of *Macrophomina phaseolina*. The thermal death range of *Verticillium albo-atrum* microsclerotia was determined by Nelson and Wilhelm (1958). Subbarao and Hubbard (1996) reported that number of *Verticillium dahliae* microsclerotia in soil was significantly reduced after incubation at 35^{0}C for 45 days. If high soil temperature affects the survival or germinability and infectivity of microsclerotia of *C. ilicicola*, the

high summer temperatures experienced in Louisiana may be a factor in determining the numbers of effective microsclerotia in field soil.

The effect of soil moisture on the soil population level of *C. ilicicola* was demonstrated by Griffin *et al.* (1978). They reported that air drying of soil (0.12 to 0.38% water or about -2,000 bars) resulted in no recovery of microsclerotia from soil, but rewetting soils to near field capacity for 1 to 4 weeks resulted in partial recovery from the deleterious effects of drying (Griffin *et al.*, 1978). Thies and Patton (1970) reported that the recovery of microsclerotia from soil was greatly reduced by air-drying. Whether this was a true loss of viability or only a temporary decrease in germinability is not known.

To understand the factors influencing soybean root colonization by *C. ilicicola* and red crown rot disease development, studies were conducted with the following specific objectives:

Objective 1: To study soybean root colonization by *C. ilicicola* and subsequent red crown rot development as influenced by planting date and cultivar susceptibility.

Objective 2: To monitor the changes in *C. ilicicola* population levels in soil over time, and examine effects of temperature on *C. ilicicola* microsclerotia in soil and on soybean root colonization by this fungus.

Objective 3: To determine the effect of host age on infection of soybean roots by *C. ilicicola*.

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CHAPTER 2

COLONIZATION OF SOYBEAN ROOTS BY *CALONECTRIA ILICICOLA* AND SUBSEQUENT RED CROWN ROT DEVELOPMENT AS INFLUENCED BY PLANTING DATE, CULTIVAR SUSCEPTIBILITY, AND SOIL PATHOGEN POPULATION

Introduction

Red crown rot, caused by the fungus *Calonectria ilicicola* Boedijn and Reitsma, is an important disease of soybean, *Glycine max* (L.) Merrill (Berggren and Snow, 1989). Yield losses due to this disease have been estimated to be as high as 50% (Berggren and Snow, 1989). This fungus causes black root rot, also known as Cylindrocladium black rot, of peanut (*Arachis hypogea*) (Bell and Sobers, 1966). Foliar symptoms of red crown rot usually appear during beginning pod (R_3) to full pod (R_4) (Fehr *et al.*, 1971) soybean growth stages and include leaf chlorosis and interveinal necrosis followed by defoliation (Berggren and Snow, 1989). The fungus colonizes roots, and reddish-brown perithecia appear in the crown region of the plant coincidentally with foliar symptoms. These perithecia are diagnostic signs of the disease. Microsclerotia produced by this fungus are survival and dispersal structures which can survive several years in soil or host debris (Bell and Sobers, 1966).

Delayed planting is recommended for red crown rot management (Berggren and Snow, 1989), but the mechanism behind reduced disease severity following delayed planting is not understood. Published reports on peanut suggest a possible role for soil temperature. Bell (1967) as well as Phipps and Beute (1977) reported soil temperature as a critical environmental factor affecting development of black root rot of peanut. Black *et al.* (1984) observed that disease severity in microplots was greater when peanuts were planted on 2 May (minimum soil temperature $<18^{0}$C) than on 17 or 30 May (minimum soil temperature $\geq 18^{0}$C).

Development of both peanut black root rot (Krigsvold *et al.* 1982; Black *et al.*, 1984) as well as soybean red crown rot (Berner *et al.*, 1988) is affected by host susceptibility. Resistant peanut cultivars are not immune to *C. ilicicola*. Pathogen entry into the peanut vascular system through the taproot is prevented by periderm production in less-susceptible cultivars (Harris and Beute, 1982). Complete host resistance to red crown rot has not been demonstrated in soybean cultivars, but some are known to be less susceptible to this disease (Kim, 1994). Severity of black root rot in peanut also depends on density of *C. ilicicola* microsclerotia in soil (Phipps and Beute, 1977). Consequently, reduction of initial inoculum density through cultural methods such as crop rotation and chemical methods such as soil fumigation (Phipps, 1990) is recommended for black root rot management in peanut (Sidebottom and Beute, 1989). Taylor *et al.* (1981) described a linear relationship between peanut root colonization and field inoculum density of *C. ilicicola*. However, the relationship between soybean root colonization and inoculum density of *C. ilicicola* has not been investigated.

The primary objective of our study was to examine the effects of delayed planting on soybean root colonization by *C. ilicicola* and subsequent red crown rot symptom development on cultivars differing in susceptibility. In addition we examined the relationship between soybean root colonization and inoculum density of *C. ilicicola* in field soil and also the changes in soil population levels of this fungus for 3 consecutive years in a soybean field. Preliminary reports have been published (Kuruppu *et al.*, 1995; Kuruppu and Russin, 1996).

Materials and Methods

Field experiments were conducted during the growing seasons of 1994, 1995, and 1996 at the Louisiana State University Agricultural Center Ben Hur Research Farm in Baton Rouge, LA, in a field that had a history of red crown rot. The soil type in the field was Mhoon silty clay loam (in order Inceptisols, suborder: Aquept, in subgroup: Fluventic Haplaquepts, in family: Fine-silty, mixed nonacidic, thermic). Following are some of the chemical properties of this soil based on tests conducted by the Louisiana State University Soil Test Laboratory, Agronomy Department, Baton Rouge, LA: pH, 5.3; organic matter, 0.77%; and sum of bases 9.34 meq/100g. The following values are expressed in mg/kg: P, 175; K, 70; Ca, 1361; Mg, 251; and Na, 69. The following elements (mg/kg) were extracted with HCl and DTPA, respectively: As, 2.46 and 0.00; Cd, 0.49 and 0.35; Cu, 2.36 and 1.76; Fe, 131.59 and 159.51; Mn 15.00 and 7.75; Ni, 1.63 and 1.48; Pb, 2.39 and 2.24; Zn 12.71 and 11.63. The experimental design was a split plot with 3 planting dates spaced 3 wks apart as main plots and 2 cultivars (Sharkey and Cajun) as subplots. The first planting was on the last week of May which falls in the optimal planting time for determinant soybean cultivars in the southern United States (Boquet, 1994) and is referred to as the optimal planting date in this Chapter. Optimal planting dates were May 25, 24, and 30 in 1994, 1995, and 1996 respectively. Both cultivars exhibit a determinant growth habit and are in maturity group VI. Sharkey was rated as the most susceptible and Cajun was one of the least susceptible among 18 cultivars screened previously for red crown rot reaction in the same field (Kim, 1994). Treatments were replicated 6

times. Each plot had 4 rows that were 7.6 m long and planted on 0.76 m centers. These plots were not disrupted by tillage after each growing season to avoid pathogen distribution among plots, and both cultivars were planted in the same plots in all 3 growing seasons. Soil sampling was done approximately every 3 wks from planting until harvest and every 2-3 months during the fallow period. Approximately 500 g of soil were collected in a zig-zag manner from the inner 2 rows in each field plot using soil probes to collect soil to a depth of 10 -15 cm. Soil samples were stored in plastic bags at room temperature (25-27^{0}C) and assayed within 2-3 wks of collection. Storage at this temperature was shown to have no effect on microsclerotia germination (Roth *et al.*, 1979).

In 1994, 1995, and 1996, soil temperature at a depth of 10 cm was recorded every hour using thermistors attached to Campbell 21X microloggers (Campbell Scientific, Inc., Logan, UT). Rainfall data in 1994, 1995, and 1996 at Ben Hur were obtained from Louisiana Office of State Climatology, Louisiana State University, Baton Rouge, LA.

Determination of microsclerotia number in soil. Each soil sample was mixed well by hand and 100 g were dried at 105^{0}C for 48 hrs to determine the soil moisture content. The method of Phipps *et al.* (1976) was used with modifications to enumerate microsclerotia from soil. A 250-g portion of the soil sample was blended with 100 ml of water and the suspension was passed through nested sieves of 150 μ over 45 μ pore size. Material on the 45 μ sieve was washed with water and the soil suspension was mixed with NaOCl (0.25%) for 30 seconds. This suspension then was poured over a sieve of 45 μ pore size and washed well with 200 ml water. One ml of

this soil suspension was mixed with 100 ml of modified Phipps semi-selective medium cooled to 50°C. Basic constituents of this medium (Phipps *et al.*, 1976) included glucose, 15 g; yeast extract, 0.5 g; agar, 20 g; distilled water 1 liter. After autoclaving, each 100-ml aliquot of the medium was amended with Tergitol, 0.1 ml; thiabendazol (10 mg suspended in 50 ml water), 0.1g; chloramphenicol (1 g dissolved in 50 ml 95% ethanol), 0.5 ml; and chlorotetracycline (0.4 g disolved in 50 ml 50% ethanol) , 0.5 ml. The medium mixed with the soil suspension was poured into 5 petri plates (9 cm in diameter). These plates were incubated for 7-10 days at room temperature (25-27°C), after which the number of *C. ilicicola* colonies (Fig. 2.1) in all 5 plates was determined.

Determination of root colonization. Colonization of soybean roots by *C. ilicicola* was monitored throughout the growing season beginning 3 wks after planting. Four plants were uprooted randomly from the 2 outer rows of each plot. Root systems were washed thoroughly and cut into segments 1 cm in length. When plants were small, the entire root system was assayed. When plants were larger, 50 segments were selected at random from the lateral roots and 20 segments were selected from taproots. In 1994, lateral and taproots were assayed separately only for the first (week 24) and last (week 34) sampling. In 1995 and 1996, tap and lateral roots were always assayed separately. Root segments were surface- sterilized in 0.25% NaOCl for 30 seconds, rinsed 3 times in sterile water, and blotted on sterile filter paper. Root segments then were plated on modified Phipps medium and incubated at room temperature under continuous fluorescent light for

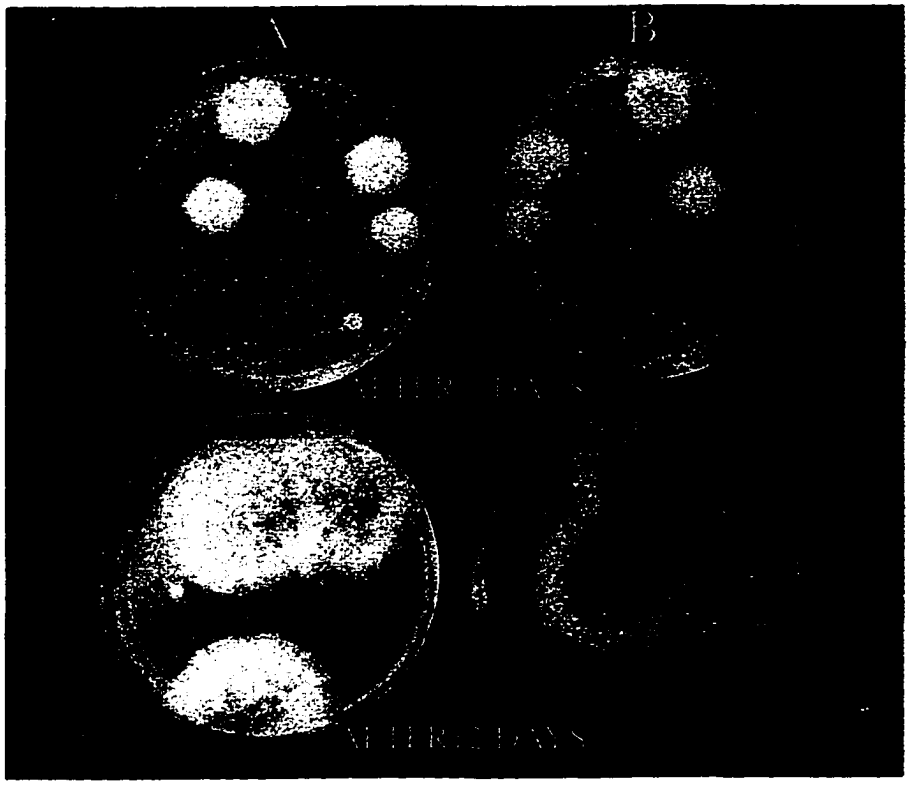

Fig.2.1. Colonies of *Calonectria ilicicola* produced on modified Phipps medium. Upper (A) and lower (B) surfaces of assay plates respectively after 7 and 12 days incubation at room temperature (25-27°C).

10-14 days. Root colonization was expressed as percentage of segments from which colonies of *C.ilicicola* were recovered.

Determination of disease incidence. Foliar symptoms, expressed as leaf chlorosis and interveinal necrosis, and/or signs (perithecia) generally appeared beginning at the R_3 growth stage. At this time the number of plants showing symptoms and signs in the inner two rows of each plot was recorded approximately every 12-14 days. The total number of plants in these rows also was recorded. Areas under disease progress curves (AUDPC) for disease incidence were calculated according to a standard formula given below:

$$AUDPC = \sum_i^{n-1}(y_i+y_{i+1}/2)\ (t_{i+1}-t_i)$$

in which y_i = disease incidence at the *ith* assessment, t_i = time (days) at the *ith* assessment, and n = total number of assessments (Shaner and Finney, 1977).

Data were analyzed using SAS General Linear Models procedure (SAS Institute, Cary, NC) to determine the main and interactive effects of planting date, cultivar, and time of sampling on disease incidence, root colonization, and *C. ilicicola* population in soil. Correlation analysis was done to examine relationships between root colonization with planting date, cultivar, and *C. ilicicola* soil population.

Results

In 1994, the weekly average soil maximum temperature was 25-27^{0}C for the 6- wk period prior to the optimal planting time (i.e., week 15-21) (Fig. 2.2). Temperatures increased rapidly after that time and were

35-39^{0}C for a 5-wk period beginning in week 33, but decreased to $\leq$ 30^{0}C over the next 4 wks (Fig. 2.2). The level of germinable microsclerotia of *C. ilicicola* in soil was low (2 colony forming units (cfu) g^{-1}soil) at planting in 1994. It increased steadily over the next several weeks and reached a maximum during week 39, then decreased during the winter (Fig. 2.2). Soil pathogen populations were similar in plots with Sharkey and Cajun cultivars until 19 wks after the first planting date (week 40) (Fig. 2.3). At that time the pathogen population in soil planted to the susceptible cultivar Sharkey increased to a level nearly twice that in soil planted to the less susceptible cultivar Cajun (23 vs. 13 cfu g^{-1}soil), and this difference remained constant through the end of the year (Fig. 2.3). In 1995, soil temperatures reached 25^{0}C by week 14, 1 wk earlier than during 1994 and were > 30^{0}C by 2 wks before optimal planting time. Soil temperature also was about 5^{0}C warmer than during 1994 (Fig. 2.2). This pattern continued through the remainder of the growing season (Fig. 2.2). Temperatures were $\geq$ 35^{0}C for 10 of 13 wks beginning in week 28 and averaged 42^{0}C and 43^{0}C during two of these weeks (weeks 33 and 36) (Fig. 2.2). Populations of *C. ilicicola* in soil initially were higher in 1995 than in 1994, and averaged 17 cfu g^{-1} across cultivars at the first planting date (week 21). However, they showed a surprising drop to $\leq$ 2 cfu g^{-1}soil by the third planting date (week 27) and remained very low ($\leq$ 3 cfu g^{-1} soil) throughout the remainder of the season. This drop in detectable pathogen population corresponded with the period during which soil maximum temperatures

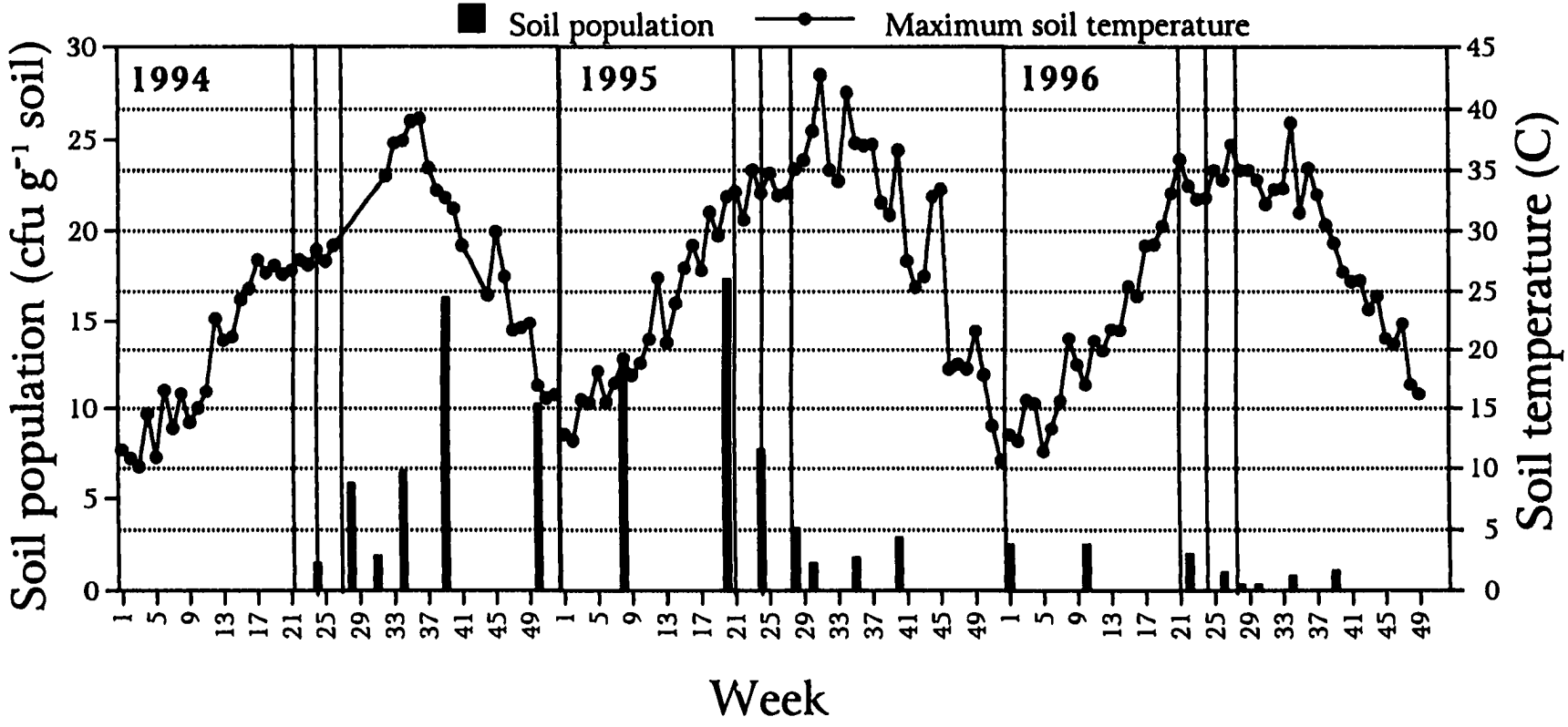

Fig.2.2. Soil population levels of *Calonectria ilicicola* and the maximum weekly averages of soil temperatures at a depth of 10 cm at the experimental site on Ben Hur Research Farm, Baton Rouge in 1994, 1995, and 1996. Three vertical lines within each year indicate three planting dates.

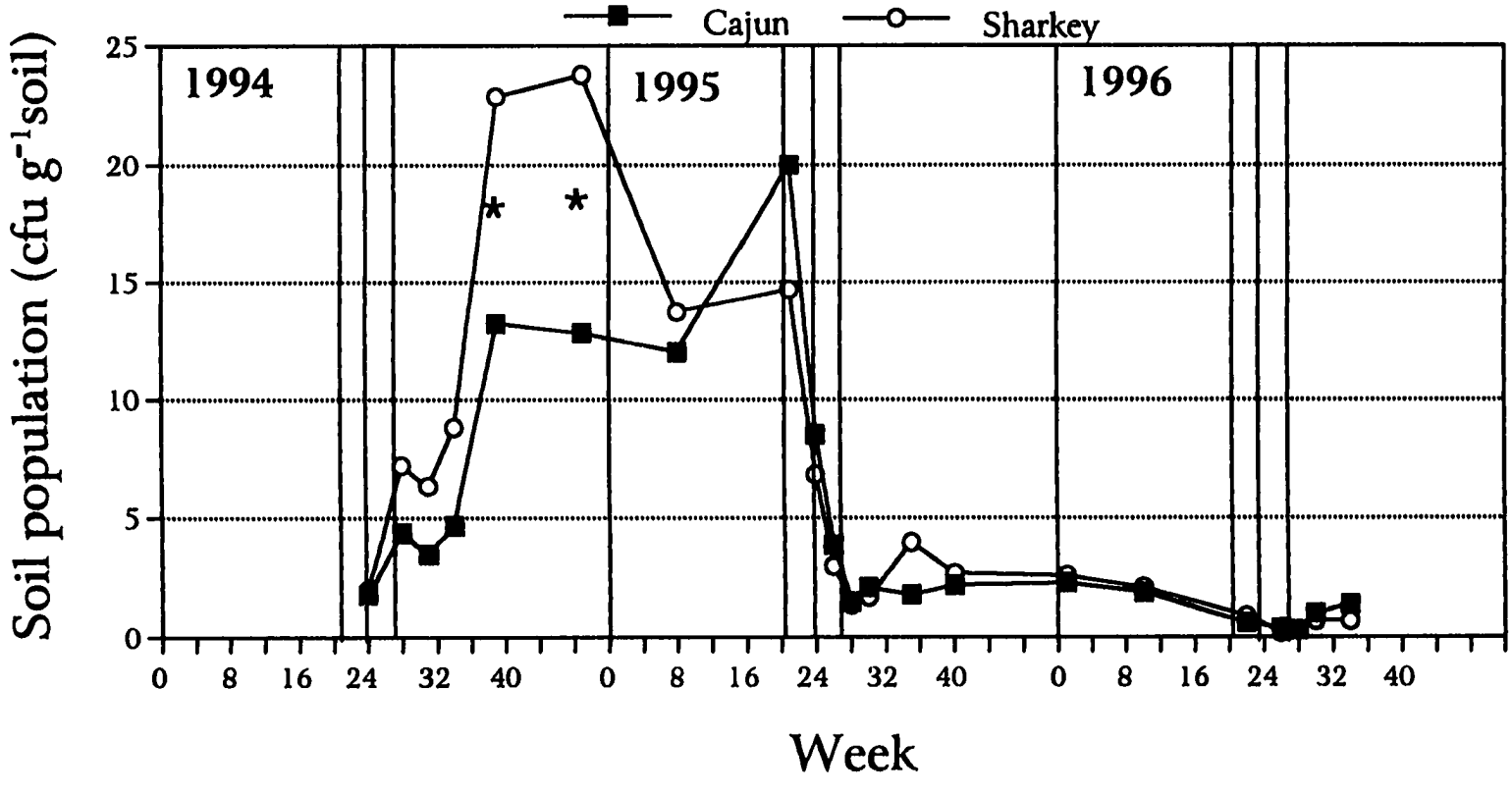

Fig.2.3. Population levels of *Calonectria ilicicola* in field soil in 1994, 1995, and 1996. Three vertical lines in each year indicate planting dates. Difference between cultivars was significant only on dates marked with asterisks, according to least squares means ($P \leq 0.05$).

approached and exceeded 40^{0}C (Fig. 2.2). The differences in soil pathogen population between plots with both cultivars no longer were detected (Fig. 2.3). Populations of *C. ilicicola* in soil remained very low during 1996, despite the fact that maximum soil temperatures were lower than those in 1995 (Fig. 2.2). The total rainfall received during the soybean planting times (weeks 20-28), as well as, the period between soybean planting and early reproductive stages (weeks 20-35), were 36.8 and 54.6 cm, respectively in 1994, 15 and 30.2 cm, respectively in 1995, and 18 and 42.9 cm , respectively, in 1996 (Fig. 2. 4).

In 1994, *C. ilicicola* soil populations correlated positively with tap, lateral, and total root colonization by *C. ilicicola*, but the correlation was far stronger for lateral than for taproots (Table 2.1). This relationship was detected in 1995 as well, but values for *r* were lower (Table 2.1). In 1996, there were positive correlations between soil population and both tap and total root colonization but not with lateral root colonization (Table 2.1).

Colonization of soybean roots was detected as early as one week after planting (data not presented). Root colonization generally was not accompanied by visible discoloration or necrosis, at least during early plant stages. In 1994, total root colonization across both cultivars at the optimal planting date averaged nearly 10% by 3 wks after planting (week 24) and remained at that level through week 28 (Fig. 2.5). It increased steadily thereafter and reached nearly 30% by week 34. Planting delays of 3 or 6 wks resulted in much lower levels of root colonization ($\leq$ 4%) by *C. ilicicola* (Fig. 2.5). Total root colonization levels did not differ between these late planting dates and did not increase as the season progressed. In

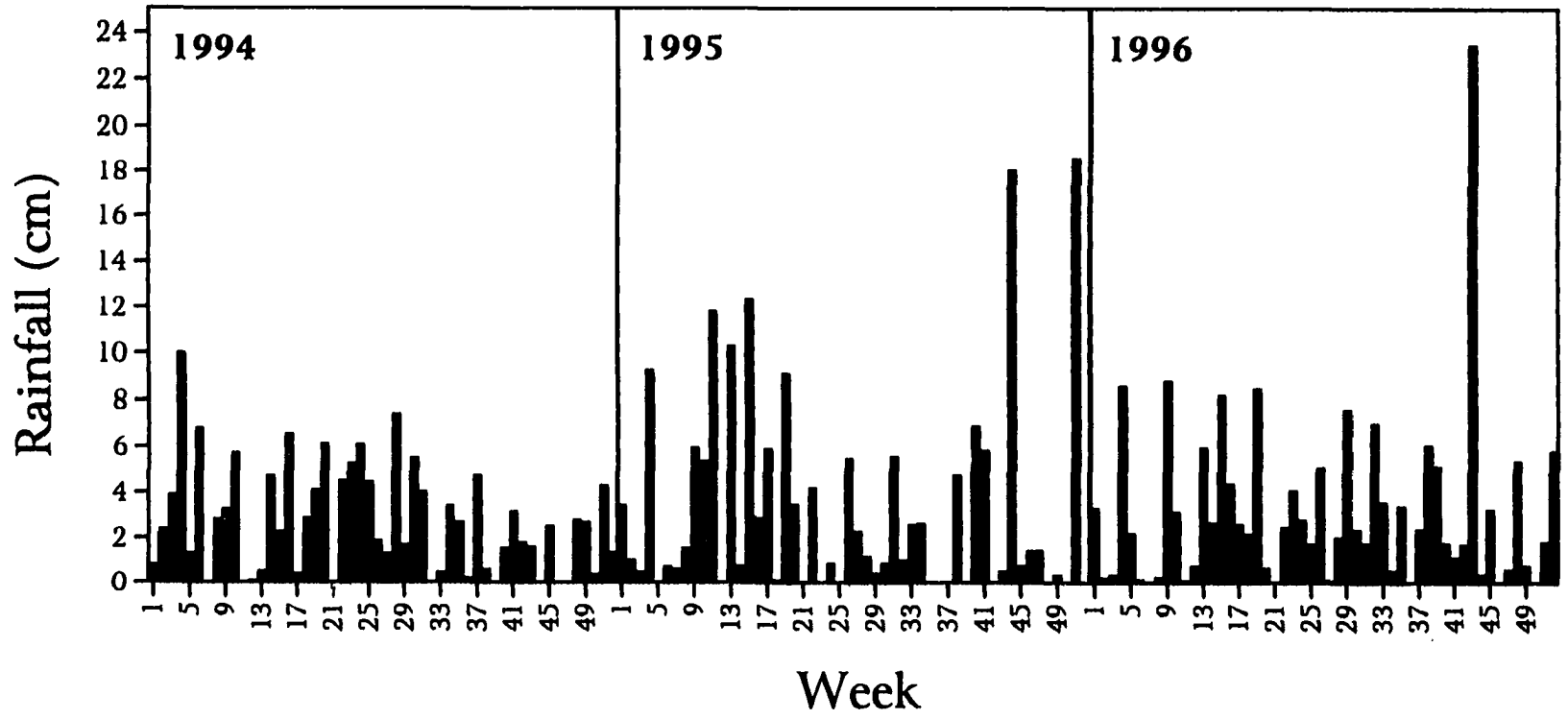

Fig.2.4. Weekly total rainfall at the experimental site on Ben Hur Research Farm, Baton Rouge in 1994, 1995, and1996.

Table 2.1. Correlations between soil population of *Calonectria ilicicola* and soybean tap, lateral, and total root colonization by *C. ilicicola* in 1994, 1995, and 1996

Factor	Year		Root colonization (%)[a]		
			Lateral	Tap	Total
Soil population[b]	1994	*r*[c]	0.7229	0.3877	0.4861
		P[d]	0.0001	0.0065	0.0001
Soil population	1995	*r*	0.2621	0.2310	0.2812
		P	0.0015	0.0053	0.0006
Soil population	1996	*r*	0.1324	0.2988	0.2610
		P	0.1719	0.0017	0.0064

[a] Averaged across cultivars and sampling dates.
[b] Number of colony forming units g^{-1} soil.
[c] Pearson correlation coefficient.
[d] Prob > |R|.

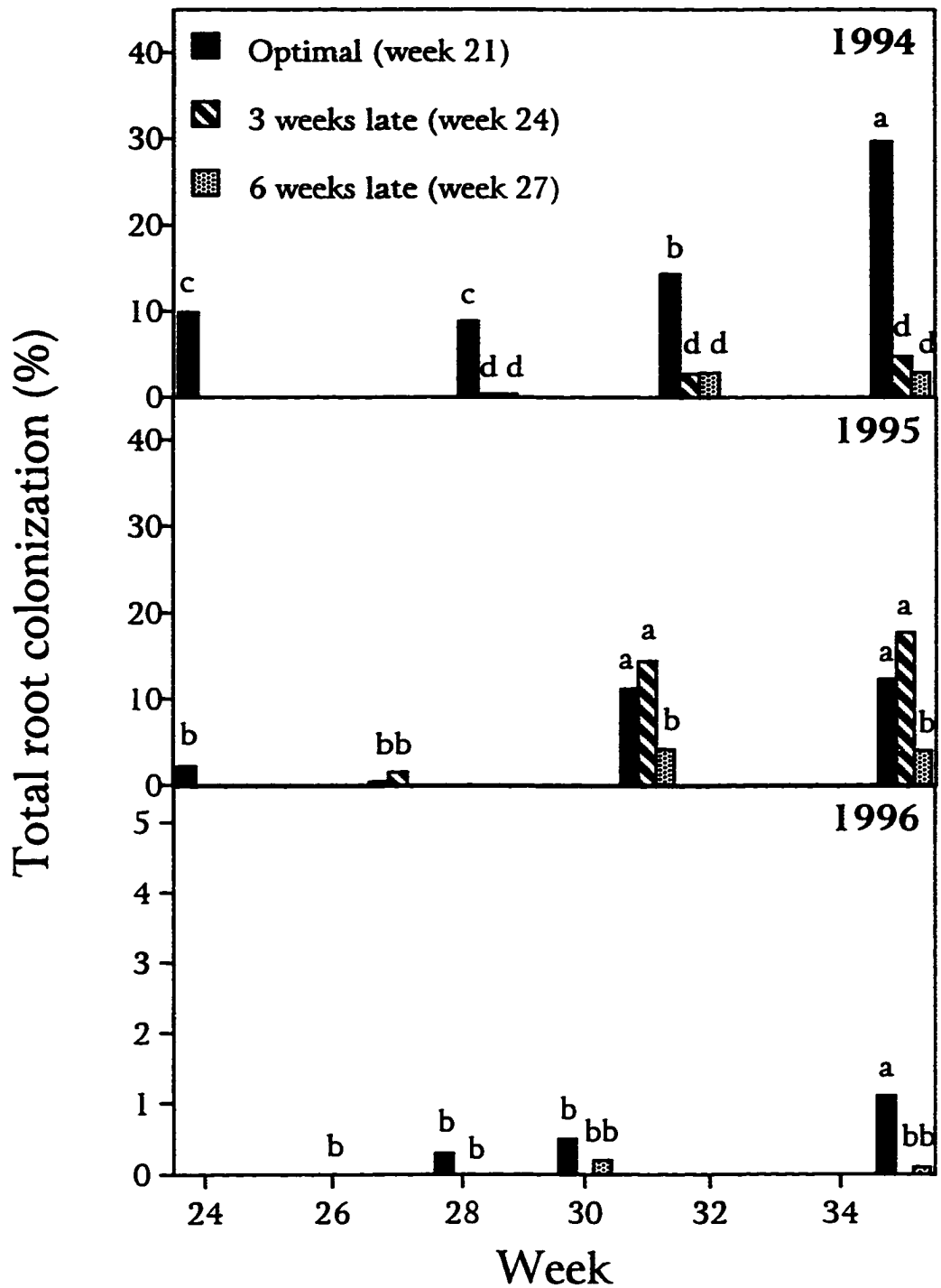

Fig.2.5. Root colonization by *Calonectria ilicicola* following 3 planting dates in 1994, 1995, and 1996. Data are expressed as means across 2 soybean culivars. Means marked with the same letter did not differ significantly in each year, according to least squares means ($P \leq 0.05$).

1995, early season root colonization across both cultivars was considerably lower (2 vs 10%) than in 1994 (Fig. 2.5). At the optimal and 3 wks late planting dates, colonization reached about 12% by week 31 and remained at that level through the end of the season. Beginning in week 31, root colonization in the last planting was less than that in earlier plantings (Fig. 2.5). Soybean root colonization in 1996 was not detected until week 28 in the optimal planting and remained very low ($\leq$1%) throughout the remainder of the season (Fig. 2.5). Root colonization in late plantings was lower than that in the optimal planting only on the last sampling date (Fig. 2.5).

Following the optimal planting date in 1994, total root colonization for susceptible cultivar Sharkey was greater than that for the less susceptible cultivar Cajun only during the first 7 wks after planting, i.e., during week 24 and 28 (Table 2.2). No differences in total root colonization were detected between these cultivars at later sampling dates. When colonization of tap and lateral roots were examined separately, only lateral root colonization differed at week 24, the earliest sampling date (Table 2.3). This difference was not detected at week 34. No differences in tap, lateral, and total root colonization were detected between cultivars when planting was delayed 6 wks (Table 2.2 and 2.3). Early root colonization did not differ between these cultivars in 1995 and 1996 (Table 2.4 and 2.5).

Symptoms and signs of red crown rot were observed only in 1994. Disease first was detected in Sharkey 10 wks after planting regardless of planting date (data not presented). Plants always were in early R stages

Table 2.2. Total root colonization by *Calonectria ilicicola* of soybean cultivars Cajun and Sharkey following planting at optimal (week 21) or delayed dates in Baton Rouge, LA in 1994

Planting date	Cultivar	Total root colonization (%)[a]			
		Week			
		24	28	31	34
Optimal (week 21)	Cajun	4.6	4.5	16.3	29.7
	Sharkey	15.0 **	13.3 *	12.3	33.9
3 weeks late (week 24)	Cajun	-	0	1.6	1.6
	Sharkey	-	0.6	3.8	7.8
6 weeks late (week 27)	Cajun	-	0.3	1.5	1.1
	Sharkey	-	0.3	4.3	4.7

[a] Asterisks indicate a significant (* = $P \leq 0.05$; ** = $P \leq 0.01$) difference between cultivars at each sampling date, according to least squares means.

Table 2.3. Tap and lateral root colonization by *Calonectria ilicicola* of soybean cultivars Cajun and Sharkey following planting at optimal or delayed dates in 1994

Planting date	Cultivar	Root colonization (%)[a]			
		Tap		Lateral	
		Week		Week	
		24	34	24	34
Optimal (week 21)	Cajun	13.3	20.5	2.9	30.7
	Sharkey	23.5	30.5	13.8*	35.0
3 weeks late (week 24)	Cajun	-	0.8	-	0.7
	Sharkey	-	6.7	-	8.3
6 weeks late (week 27)	Cajun	-	2.5	-	0.7
	Sharkey	-	6.7	-	4.3

[a] Asterisks indicate a significantly (* = $P \leq 0.05$; ** = $P \leq 0.01$) difference between cultivars at each sampling date, according to least squares means.

Table 2.4. Total root colonization by *Calonectria ilicicola* of soybean cultivars Cajun and Sharkey following planting at optimal (week 21) or delayed dates in Baton Rouge, LA in 1995 and 1996

		1995			
		Week			
		24	28	30	35
Optimal (week 21)	Cajun	1.8	2.2	9.9	9.7
	Sharkey	2.5	3.1	12.3	14.7
3 weeks late (week 24)	Cajun	-	0.7	9.4	11.3
	Sharkey	-	2.3	19.3 *	24.0 **
6 weeks late (week 27)	Cajun	-	0	4.5	4.5
	Sharkey	-	0	3.8	3.5

		1996			
		Week			
		26	28	30	34
Optimal (week 21)	Cajun	0	0.2	0.4	0.5
	Sharkey	0	0.4	0.6	1.7*
3 weeks late (week 24)	Cajun	-	0	0	0
	Sharkey	-	0	0	0
6 weeks late (week 27)	Cajun	-	0	0	0.2
	Sharkey	-	0	0.4	0

[a] Within years and planting dates, asterisks indicate a significant (* = $P \leq 0.05$; ** = $P \leq 0.01$) difference between cultivars at each sampling date, according to least squares means.

Table 2.5. Tap and lateral root colonization by *Calonectria ilicicola* of soybean cultivars Cajun and Sharkey following planting at optimal or delayed dates in 1995 and 1996

		1995							
		Tap				Lateral			
		Week				Week			
		24	26	30	35	24	26	30	35
Optimal (week 21)	Cajun	0.5	0.5	22.5	20.8	4.2	2.5	5.0	6.7
	Sharkey	2.9	3.9	14.2	19.2	1.9	1.9	11.7	13.0
3 weeks late (week 24)	Cajun	-	0	22.5	16.7	-	1.7	4.7	14.2
	Sharkey	-	2.2	23.3	32.5 *	-	5.0	18.0**	20.7
6 weeks late (week 27)	Cajun	-	-	7.5	7.5	-	-	3.3	3.3
	Sharkey	-	-	8.3	7.5	-	-	2.0	2.0

		1996							
		Tap				Lateral			
		Week				Week			
		26	28	30	34	26	28	30	34
Optimal (week 21)	Cajun	0	0	0.5	2.2	0	0	0.3	0
	Sharkey	0	0	1.1	2.2	0	0.7	0.3	1.0
3 weeks late (week 24)	Cajun	-	0	0	0	-	0	0	0
	Sharkey	-	0	0	0	-	0	0	0
6 weeks late (week 27)	Cajun	-	-	0	0	-	-	0	0.3
	Sharkey	-	-	1.4	0	-	-	0	0

[a] Within years and planting dates, asterisks indicate a significantly (*=$P \leq 0.05$; **=$P \leq 0.01$) difference between cultivars at each sampling date, according to least squares means.

when symptoms were detected. Disease incidence in Sharkey was high and averaged about 20% following optimal planting, whereras disease in less susceptible Cajun was significantly lower all sampling dates (Fig. 2.6). When planting was delayed 3 wks, disease incidence in Sharkey was lower than that following optimal planting and was greater than that in Cajun only at the last 3 sampling dates. There was no significant difference in disease incidence between cultivars when planting was delayed 6 wks. Incidence of red crown rot was consistently low ($\leq$ 7%) in Cajun regardless of planting date (Fig. 2.6). Area under disease progress curve (AUDPC) values for Sharkey showed a stepwise decrease with delay in planting, whereas AUDPC values for Cajun remained low and did not decrease in response to planting date (Fig. 2.7).

Discussion

Red crown rot disease of soybean is caused by the soil borne fungus *C. ilicicola*, which enters the soybean plant through the root system during vegetative soybean growth stages. In this Chapter, the term 'root colonization' refers to growth of the fungus within soybean root tissues (Agrios, 1997). Results of our study show that soybean root colonization by *C. ilicicola* was not generally accompanied by visible root discoloration. Taylor *et al.* (1981), also isolated the pathogen from soybean roots with little or no root rot. These observations suggest that root discoloration can not be taken as a disease symptom of red crown rot of soybean. Above ground symptoms and signs of red crown rot appeared only during the reproductive stages of soybean plants in the field.

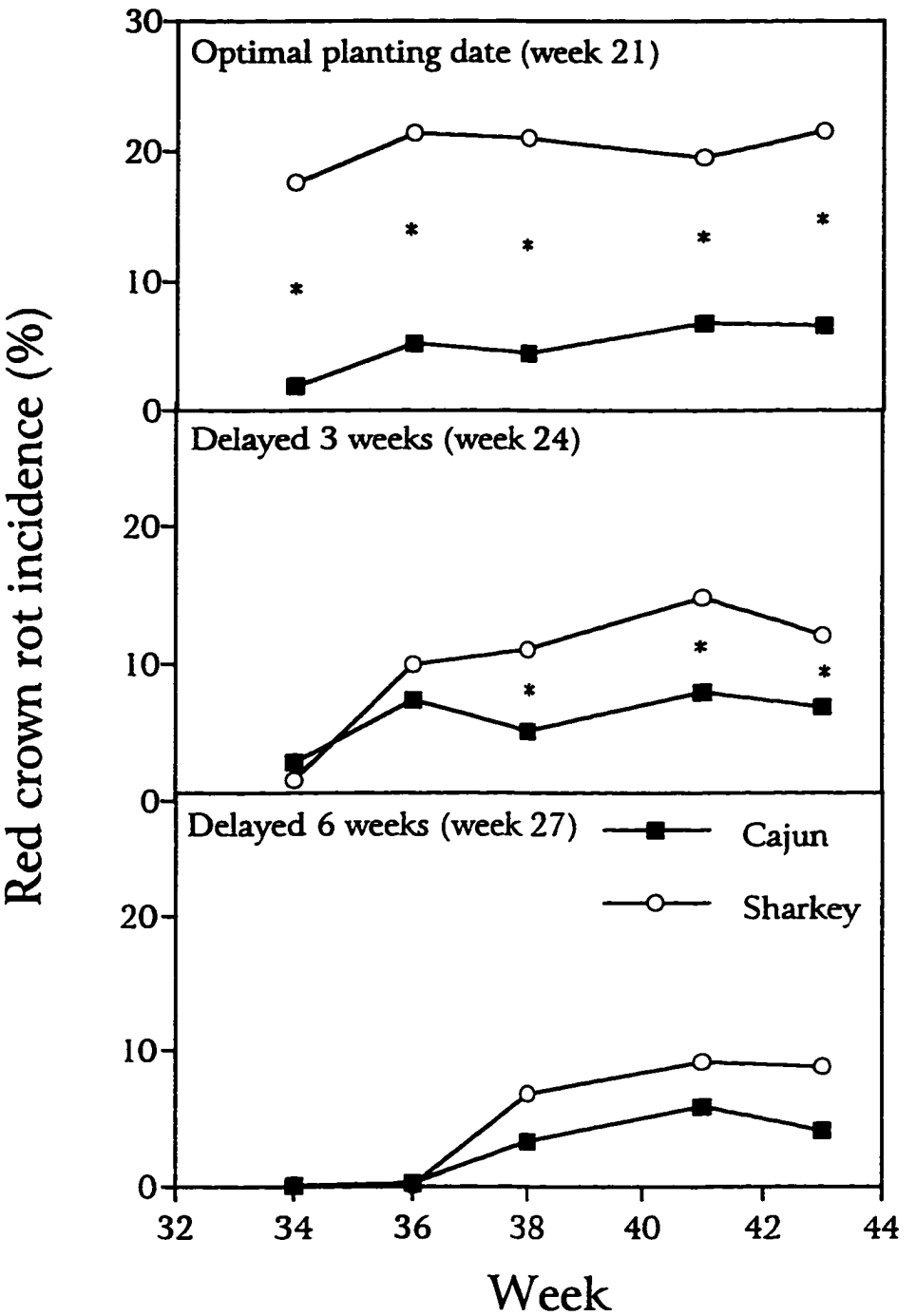

Fig.2.6. Incidence of red crown rot in 2 soybean cultivars planted in 1994. Within each planting date, asterisks indicate significant ($P \leq 0.05$) difference in disease incidence between cultivars at each evaluation time according to least squares means.

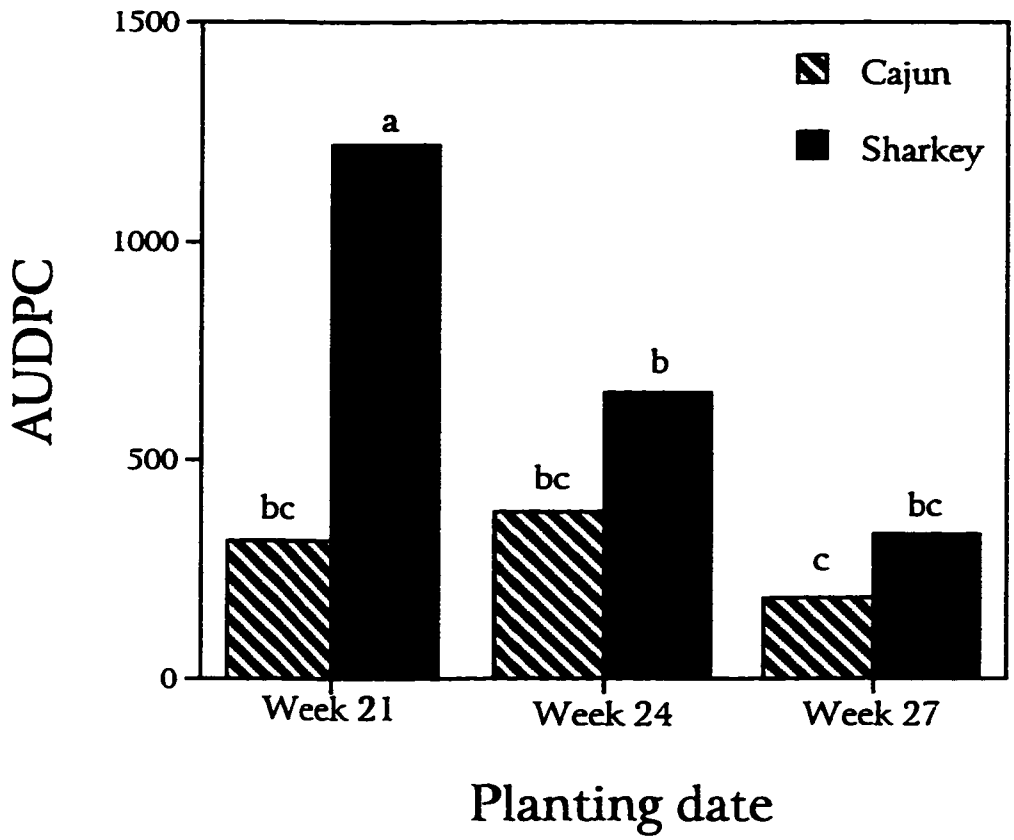

Fig.2.7. Areas under disease progress curves (AUDPC) for red crown rot incidence on 2 soybean cultivars planted at an optimal date (week 21), 3 weeks late (week 24), and 6 weeks late (week 27) in 1994 growing season. Bars with same letter did not differ significantly according to least squares means ($P \leq 0.05$).

Published reports on peanut suggest a possible role for soil temperature. Bell (1967) as well as Phipps and Beute (1977) reported soil temperature as a critical environmental factor affecting development of black root rot of peanut. Black *et al.* (1984) observed that disease severity in microplots was greater when peanuts were planted on 2 May (minimum soil temperature <18 C) than on 17 or 30 May (minimum soil temperature ≥18 C).

Several investigators have reported that red crown rot incidence on soybean was reduced following delayed planting (Russin *et al.*, 1985; Berner *et al.*, 1988) and delayed planting therefore is, recommended for red crown rot management (Berggren and Snow, 1989). The mechanism behind reduced disease severity following delayed planting is not understood. Results from our study support these findings, but more importantly provide useful information about the mechanism behind this reduction. Analysis across both soybean cultivars revealed that initiation of root colonization was delayed and final root colonization levels were reduced when planting was delayed by 3 or 6 wks. Separate analysis for each cultivar provided an additional insight into this reduction. As expected, red crown rot incidence on the susceptible cultivar Sharkey as much greater than that on the less susceptible cultivar Cajun which planted at the optimal planting date in 1994. However, root colonization levels differed between these 2 cultivars only during the first few weeks following optimal planting, not later in the season when symptoms were evident. This suggests that the red crown rot symptom levels, which appeared at the end of the season, depended on root colonization that occurred during a short period of about

7 wks after planting. These results suggest that early root colonization by *C. ilicicola* is critically important for the later development of red crown rot symptoms and signs in soybean. The very low level of early root colonization and absence of red crown rot symptoms, even in the susceptible Sharkey, in 1995 and 1996 further support this suggestion. Tap and lateral root colonization were considered separately in both cultivars during 1994, the year red crown rot disease symptoms were evident. Taproots were equally colonized, but the lateral root colonization was different between cultivars during this short period following optimal planting. These results indicate the importance of early colonization of the whole root system (tap as well as lateral roots) of soybean plant for subsequent development of red crown rot.

Results of this study indicate that field conditions are conducive for soybean root colonization by *C. ilicicola* only during a short period in the beginning of summer. Therefore it is advisable to avoid this period when planting susceptible soybean cultivars in fields with a history of red crown rot. However, delays in planting also may cause soybean yield reduction apart from that caused by red crown rot. Growers should choose carefully the planting times for susceptible cultivars in order to balance potential yield losses due to extremely delayed planting against yield increases due to disease reduction.

Low disease incidence in Cajun regardless of planting date shows the importance of planting less susceptible soybean cultivars to manage red crown rot and stresses the importance of developing resistant cultivars. Results of this study suggest a relationship between reduced disease in

Cajun and its ability to limit early lateral root colonization by *C. ilicicola*. Harris and Beute (1982) reported that in less susceptible peanut cultivars reduce pathogen entry into the vascular system through periderm production in the taproot. However, histological changes occur in soybean roots in response to this pathogen have not yet been examined. Therefore, histological studies of soybean root colonization by *C. ilicicola* may be useful to enhance breeding and selection of soybean cultivars resistant to red crown rot.

The low level of average pathogen population in this field at the beginning of the 1994 growing season may be attributable to vigorous land preparation done prior to the initiation of the field study. These field plots were not disturbed during the field study which was continued for 3 years, in order to monitor the changes in soil pathogen population levels. The populations of *C. ilicicola* in soil planted to soybean cultivars differing in susceptibility was not consistent during the 3 consecutive growing seasons in this field. A higher level of *C. ilicicola* in the soil of plots planted to the susceptible Sharkey was detected towards the end of 1994, probably caused by the deterioration of the more heavily colonized Sharkey lateral roots towards the end of the year. However, this difference disappeared during spring 1995, and the pathogen population was not different in the soil plots planted to these 2 cultivars during the next growing seasons. Also, the pathogen population level in soil during the early summer of the 1995 took a dramatic drop and continued to be low even in the presence of a susceptible host. Black *et al.* (1984) showed that the mean population density was lower following monoculture for 4 years with a resistant peanut

cultivar than with a susceptible cultivar. However, Black and Beute (1985) did not observe an increase in inoculum density of *C. ilicicola* after two cycles of 2 to 3 months monoculture (greenhouse) of peanut or soybean. In our study, continuous cultivation of a susceptible host for 3 consecutive years did not result in increased *C. ilicicola* population levels in field plots. This suggests that factors other than the presence of susceptible soybean may influence the soil population level of *C. ilicicola* in soybean fields.

Understanding the relationship between soil population levels of *C. ilicicola* and root colonization is important in the study of disease development and management of red crown rot disease in soybean. Although there was a weak but positive correlation between pathogen populations in the soil and taproot colonization in all 3 years, a strong positive correlation between lateral root colonization and pathogen populations in soil was detected in 1994, the only year in which red crown rot symptoms were expressed in soybean plants. This was likely caused by deterioration of heavily colonized lateral roots (towards the end of the year), which contributed to the increase in *C. ilicicola* population in field soil. Reduced initial root colonization in 1995, in spite of high pathogen populations (average of 17 cfu g^{-1}soil) during planting time, was unexpected after detecting such a strong positive correlation between soil pathogen level and root (mainly lateral root) colonization in the previous year. Black and Beute (1984) and Black *et al.* (1984) suggested that factors other than the density of microsclerotia in soil affect the severity of black root rot of peanut. It also was reported that black root rot is largely affected by soil temperature and soil moisture (Phipps and Bute, 1977; Pataky *et al.*, 1983).

According to Phipps and Beute (1977), *C. ilicicola* was most aggressive in peanut soils with moisture levels near field capacity, but was less aggressive when soil was allowed to dry to near permanent wilting point. In our study, reduced early season root colonization in 1995 in spite of high levels of soil pathogen population, may be attributable to high soil temperatures and low rainfall experienced during early growing season. Environmental conditions may not only affect the process of soybean root infection by *C. ilicicola*, but also the viability of *C. ilicicola* microsclerotia. As early soybean root colonization is critical for the development of red crown rot, the effects of these environmental factors on early season soil pathogen levels as well as early season root colonization by the pathogen might be important in development of red crown rot in soybean. Subsequent chapters will address effects of temperature on soil population levels of *C. ilicicola* and soybean root colonization by this pathogen and some of the questions raised during this field study as well.

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CHAPTER 3

SOIL TEMPERATURE EFFECTS ON MICROSCLEROTIA OF *CALONECTRIA ILICICOLA*, AND SOYBEAN ROOT COLONIZATION BY THIS FUNGUS

Introduction

Red crown rot of soybean (*Glycine max* (L.) Merrill) was first reported in Louisiana in 1976 and soon was recognized as an important soybean disease in that state (Berner *et al.*, 1988). The causal agent for red crown rot is the soil borne fungus *Calonectria ilicicola* Boedijn and Reitsma (Rowe *et al.*, 1973; Crouse *et al.*, 1993). *Calonectria ilicicola*, previously known as *Calonectria crotalariae* (Crouse *et al.*, 1993), is the perfect stage of *Cylindrocladium parasiticum*, previously known as *Cylindrocladium crotalariae*. In 1966, Bell and Sobers described this fungus as the causal agent for black root rot, also known as Cylindrocladium black rot, of peanut (*Arachis hypogea*). This fungus produces both conidia and ascospores, but roles for these spores in the disease cycle are not known. Microsclerotia are the survival and dispersal structures produced by the fungus and they survive several years in soil or on host debris (Bell and Sobers, 1966). Foliar symptoms of red crown rot usually appear during beginning pod (R_3) to full pod (R_4) (Fehr *et al.*, 1971) soybean growth stages and include leaf chlorosis and intervenal necrosis followed by defoliation (Berggren and Snow, 1989). Reddish-brown perithecia, which are the diagnostic signs of red crown rot, appear in the crown region of the plant beginning in the early reproductive stages (Berggren and Snow, 1989).

Delays in planting reduce incidence of soybean red crown rot (Russin *et al.* 1985; Berner *et al.* 1988). Recommended disease management strategies in Louisiana include delayed planting and use of less susceptible cultivars (Berner *et al.*, 1988; Berggren and Snow, 1989). However, delayed planting is the management strategy of choice for red crown rot because of

lack of resistant cultivars. The mechanism behind reduced disease incidence following delayed planting is not clear. It may be caused by escaping the most conducive field conditions for root colonization by the pathogen, by reduced number of infective microsclerotia in response to increasing soil temperature (Kuruppu and Russin, 1997), or by the cumulative effect of both phenomena. Bell (1967) and Phipps and Beute (1977) reported that soil temperature is the most important environmental factor influencing the development of black root rot of peanut. The effect of temperature on soybean root colonization by *C. ilicicola* has not been investigated.

According to Phipps and Beute (1977) and Harris and Beute (1982), disease severity in susceptible and resistant peanut cultivars may depend on microsclerotia densities in soil. Consequently, factors affecting the survival of fungal microsclerotia in field soil may be of critical importance in the development of black root rot in peanut, as well as, red crown rot in soybean. Available data suggest that effective inoculum density of *C. ilicicola* in peanut fields is fully or partially controlled by temperature and soil moisture (Phipps and Beute, 1977; Griffin *et al.*, 1978, Pataky and Beute, 1983). These authors also suggested that seasonal as well as diurnal fluctuations in these 2 factors might influence fungal propagule survival, which in turn may affect the incidence and severity of black root rot of peanut. However, temperature and soil moisture effects on survival of *C. ilicicola* microsclerotia in the heavy alluvial soils common to Louisiana soybean fields have not been studied.

The objectives of this study were to examine effects of temperature on a) survival of *C. ilicicola* microsclerotia in a heavy alluvial soil and b)

soybean root colonization by *C. ilicicola*. Preliminary reports have been published (Kuruppu and Russin, 1996; Kuruppu and Russin, 1997).

Materials and Methods

Soil for all experiments was collected from the Louisiana State University Agriculture Center Ben Hur Research Farm, Baton Rouge in a field with no history of red crown rot. The soil type in the field was Mhoon silty clay loam (in order Inceptisols, suborder: Aquept, in subgroup: Fluventic Haplaquepts, in family: Fine-silty, mixed nonacidic, thermic). Preliminary tests showed that this soil was free of *C. ilicicola*. Soil was stored in plastic bins until used. Soil temperatures used in this Chapter were collected during the delayed planting field study conducted at the Louisiana State University Agriculture Center Ben Hur Research Farm, Baton Rouge.

Soil infestation. Soil infestation was accomplished using isolate SG 915 of *C. ilicicola* (Kim, 1994). Fungus cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 25^{0}C for 6 wks. Mycelia in agar were blended with distilled water for 1 min at low speed (speed 1) in a Waring commercial laboratory blender. The slurry was poured through nested sieves of 425 μ pore size over 150 μ pore size. Material on the 150 μ sieve was washed under a stream of water to dislodge and remove hyphal fragments. Microsclerotia then were suspended in distilled water, enumerated, and mixed with soil. To avoid difficulties in separating microsclerotia from residual culture medium due to clogging, sieves with larger pore sizes (425 μ over 150 μ) were used to extract microsclerotia

from cultures than those (150 μ over 45 μ) used to extract microsclerotia from soil.

Determination of microsclerotia number in soil. Soil (200 g) was blended with 100 ml of water in a Waring commercial laboratory blender at low speed (speed 1) and the suspension was poured through nested sieves of 150 μ over 45 μ pore size. Material on the 45 μ sieve was washed with water to make a soil suspension and then mixed with NaOCl (0.25%) for 30 sec. This suspension was poured through a 45 μ sieve and the material collected on the sieve was combined with 200 ml water. One ml of this soil suspension was added to 100 ml modified Phipps medium (Phipps *et al.*, 1976) that had cooled to 50^{0}C. This mixture then was poured into 5 petri plates (9 cm in diameter). Plates were incubated for 7-10 days at room temperature (25-27^{0}C), at which time, the number of colonies representing number of germinable microsclerotia in all 5 plates was determined. A 50-g portion of the soil sample was dried at 105^{0}C for 48 hr to determine the soil moisture content and soil dry weight.

Determination of root colonization. Soybean roots were freed from soil by gentle washing under a stream of water. Tap and lateral roots were separated and then cut into segments 1 cm in length. From these, 20 taproot and 50 lateral root segments were selected at random. These root segments were surface sterilized in 0.25% NaOCl for 30 sec, rinsed 3 times in sterile water, and blotted on sterile filter paper. Root segments then were plated on modified Phipps medium and incubated at room temperature (25-27^{0}C) under continuous fluorescent light for 10-14 days.

Root colonization was expressed as percentage of segments from which colonies of *C. ilicicola* were recovered.

Minimum number of *C. ilicicola* microsclerotia required for soybean root colonization. A preliminary experiment was conducted to determine the minimum number of *C. ilicicola* microsclerotia produced on PDA in the laboratory that were required for colonization of soybean tap and lateral roots in greenhouse tests. Concentration of the microsclerotia suspension was adjusted and then mixed with soil to achieve infestation densities of 25, 50, and 75 microsclerotia g^{-1} soil. Each density was replicated 5 times. One kilogram of infested soil was added to pots (12 cm in diameter). Seeds of the soybean cultivars Sharkey and Cajun were planted in infested soil (1 seed per pot) and plants were allowed to grow in a greenhouse. Plants were harvested after 8 wks and colonization of lateral and taproots was determined for each cultivar at all infestation levels according to procedures described previously.

Germination of microsclerotia of *C. ilicicola* and subsequent colony development on nutrient medium. Effects of temperature on *C. ilicicola* microsclerotia germination and subsequent colony formation on nutrient medium were determined in a laboratory study. Microsclerotia of *C. ilicicola* isolate SG 915 were collected from PDA plates and inoculated singly to the center of each petri plates containing PDA. These plates were incubated at 20, 25, 30, 35, and 40^{0}C. Temperatures were selected because they represent the range of soil temperatures in Louisiana soybean fields during the growing season (Fig. 3.7). Each treatment was replicated 6

times. Colony diameters were measured after 7 days. The experiment was conducted twice.

Effect of temperature on germinability and infectivity of *C. ilicicola* microsclerotia. Workers who have studied temperature effects on microsclerotia of this fungus used the terms 'survival', 'viability', and 'germinability' interchangeably to refer to production of fungal colonies on nutrient media after recovering microsclerotia from soil (Krigsvold and Griffin, 1975; Phipps *et al.*, 1976; Griffin *et al.*, 1978; Roth *et al.*, 1979; Diamonde and Beute, 1981; Pataky and Beute, 1983; Black and Beute, 1984; Sidebottom and Beute, 1989). However, the questions raised would be: 1) do microsclerotia that produce colonies on nutrient media always germinate in the rhizosphere of soybean roots and subsequently invade and colonize root tissues; and 2) do microsclerotia that germinate in the rhizosphere and invade soybean roots always capable of producing colonies on nutrient media? In this Chapter the term 'infectivity' refers to the ability of microsclerotia to germinate in the rhizosphere of soybean roots and subsequently invade and colonize the host root tissue, which may also indicate the viability of microsclerotia. The term 'germinability' refers to the ability of microsclerotia to germinate and produce colonies on nutrient media.

The effects of temperature on the germinability and infectivity of *C. ilicicola* microsclerotia were tested in a greenhouse experiment. Field soil was infested with microsclerotia of *C. ilicicola* isolate SG 915 that were produced in the laboratory on PDA. A soil infestation level of 90 microsclerotia g^{-1} soil was chosen to ensure sufficient root colonization at

all inoculation temperatures. One kilogram of soil in each plastic bag was infested with *C. ilicicola* microsclerotia using the procedure described above. The amount of water that should be added to bring this soil to field capacity was predetermined and was added to soil in plastic bags, then the weight of each bag was recorded. These bags of soil were incubated at 20, 25, 30, 35, and 40^{0}C for 1, 2, 3, or 6 wks. Soil moisture level was maintained at field capacity during incubation by adding enough water to each bag to bring back to its initial weight every 2 days. Each temperature treatment was replicated 5 times. At the end of each incubation period, the number of germinable microsclerotia in soil incubated at each temperature was assayed using the method described previously. This experiment was conducted twice.

To determine the infectivity of microsclerotia in infested soil incubated at these different soil temperatures for different durations, seeds of the soybean cultivar Sharkey were planted into 12-cm diameter plastic pots (1 seed per pot) containing this soil. Each treatment was replicated 5 times. Plants were allowed to grow in a greenhouse at 25 $\pm$ 5^{0}C and plants were harvested after 8 wks. Tap and lateral root colonization levels were assayed separately using the method described previously. Soil population levels were assayed again at the end of the experiment using the method described previously. This experiment was conducted twice.

Data were analyzed using SAS General Linear Models procedure (SAS Institute, Cary, NC). Main and interactive effects of temperature and duration on germinability and infectivity of *C. ilicicola* microsclerotia were determined.

Temperature effects on root colonization. Experiments to determine the effect of temperature on soybean root colonization by *C. ilicicola* were conducted in growth chambers using a photoperiod of 14 hr light and 10 hr darkness. Average intensity of photosynthetically active radiation inside growth chambers was 39 μE m^2 sec^{-1}. Day temperatures used were 20, 25, 30, 35, and 40^{0}C and night temperatures were 15, 20, 25, 30, and 35^{0}C, respectively. These temperature regimes were selected because they represent the range of day and night temperatures in Louisiana soybean fields during the growing season (Fig. 3.7). A single temperature regime was assigned to each growth chamber.

The effect of soil temperature on invasion of soybean roots by *C. ilicicola*, which is the initial stage of root infection (Agrios, 1997), was examined. Soil was mixed well with an enumerated suspension of *C. ilicicola* microsclerotia to achieve infestation density of 50 microsclerotia g^{-1} soil. Infested soil (800 g) was added to 12-cm-diameter plastic pots. Seeds of soybean cultivar Sharkey were planted in infested soil, and plants were allowed to grow in growth chambers at each temperature regime. Plants were watered up to field capacity once each day and were harvested after 10 days. Intact root systems were surface-sterilized in 0.1% NaOCl as described previously and plated on petri dishes (1 root system per petri dish) containing Phipps medium. Production of *C. ilicicola* colonies on modified Phipps medium by root systems were examined.

The effect of soil temperature on the extent of root colonization was examined. Soil was infested with *C. ilicicola* microsclerotia to achieve infestation densities of 40, 80, and 120 microsclerotia g^{-1}soil, as described

previously. Each inoculum level was replicated 5 times. A single, pregerminated seed of the soybean cultivar Sharkey was planted in infested soil in 10-cm-diameter plastic pots. Plants were then allowed to grow in growth chambers at each temperature regime and watered up to field capacity once each day. Plants were harvested after 4 wks. The percentage of tap and lateral root segments colonized was determined using the method described previously and was expressed as percentage of tap and lateral root colonization. This experiment was conducted twice. Data were analyzed using SAS General Linear Models procedure (SAS Institute, Cary, NC) to determine the main and interactive effects of temperature and inoculum density on soybean root colonization by *C. ilicicola*.

Results

Effect of temperature on germinability and infectivity of *C. ilicicola* microsclerotia. In the preliminary experiment, colonization of soybean taproots was between 20-30% and did not differ in response to *C. ilicicola* microsclerotia density in soil (Fig. 3.1). Lateral root colonization increased significantly as inoculum level increased to 50 microsclerotia g^{-1} soil, but did not increase further at 75 microsclerotia g^{-1} soil (Fig. 3.1). Both tap and lateral root colonization did not differ between cultivars. These results suggested that an infestation level of >50 microsclerotia g^{-1} soil would give considerable root colonization (at least 20%) in soybean plants grown in a greenhouse at 25 $\pm$ 5°C.

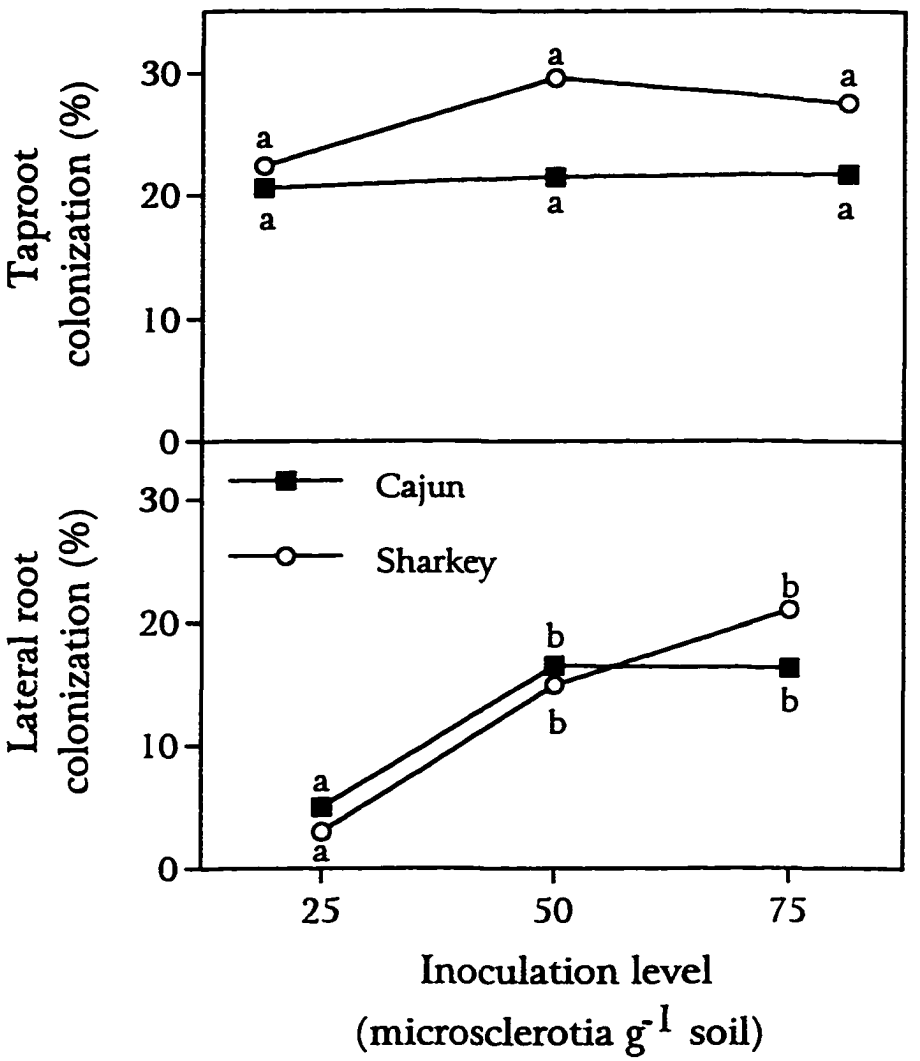

Fig.3.1. Tap and lateral root colonization of 2 soybean cultivars grown in soil infested with 3 densities of *Calonectria ilicicola* microsclerotia. For each parameter, treatment means marked by the same letter are not significant, according to least squares means ($P \leq 0.05$).

Laboratory produced microsclerotia of *C. ilicicola* germinated and produced colonies on PDA at 20, 25, 30, and 35^{0}C but not at 40^{0}C (Fig. 3.2). The fungus grew best from 25 to 35^{0}C, and maximum colony diameter occurred at 30^{0}C (Fig. 3.2). Growth at 20^{0}C was half that at 30^{0}C.

Germinability of *C. ilicicola* microsclerotia was very sensitive to incubation temperature. When averaged across all incubation durations, germinability decreased steadily as incubation temperature increased (Fig. 3.3A). Only a few of these microsclerotia germinated after incubation at 30 or 35^{0}C, and a temperature of 40^{0}C essentially was lethal (Fig. 3.3A). At lower incubation temperatures (20 or 25^{0}C), germinable microsclerotia number increased as the incubation duration increased (Fig. 3.3A). Germinable microsclerotia numbers further changed by the time soybeans were harvested after 8 wks, in soil incubated at 20, 30, and 35^{0}C (Fig. 3.3A). Germinable microsclerotia numbers further by the time soybeans were harvested after 8 wks (Fig. 3.3B). At 20^{0}C, increase in germinable microsclerotia number, still was detected as incubation duration increased, but the magnitude of difference was much less (Fig. 3.3B). By the time soybeans were harvested, no effect of incubation duration was detected on germinable microsclerotia number in soil incubated at 25^{0}C, but an opposite trend was seen at incubation temperature at 30^{0}C (Fig. 3.3B). The longer the incubation of infested soil to 30^{0}C, the lower the number of germinable microsclerotia in that soil by the time soybeans were harvested (Fig. 3.3B). The highest root colonization across all 4 durations was detected in soybeans grown in infested soil incubated at 25^{0}C (Fig. 3.4).

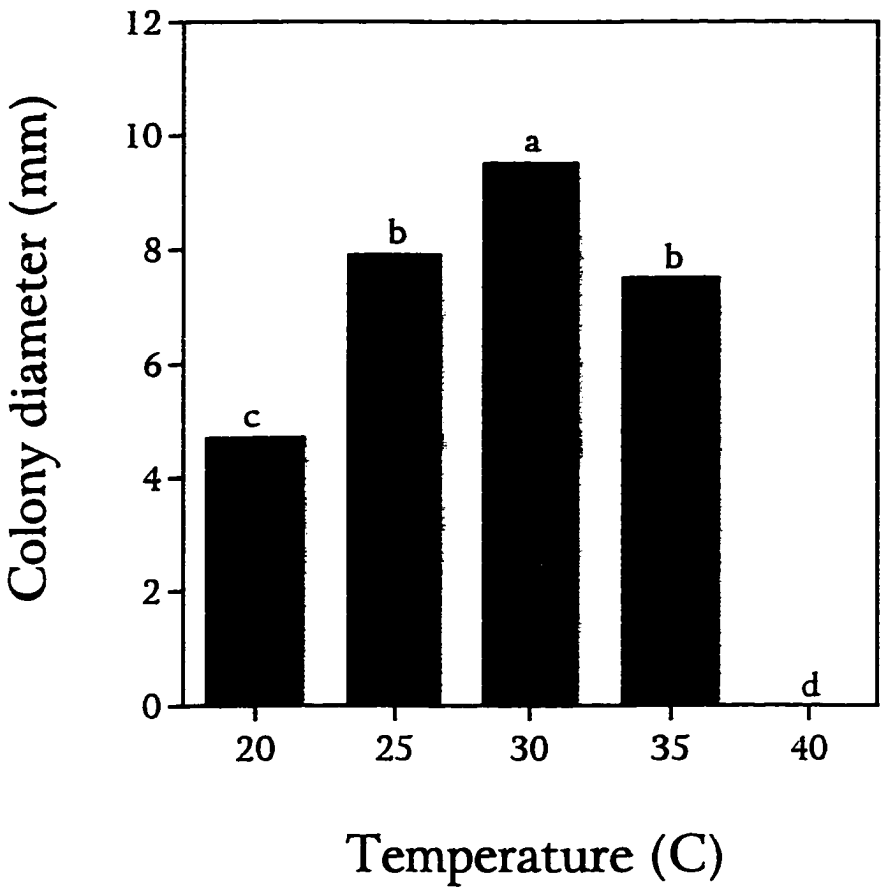

Fig.3.2. Colony diameters after 7 days for *Calonectria ilicicola* grown on potato dextrose agar at different temperatures. Means marked by the same letter are not significantly different, according to least squares means ($P \leq 0.05$).

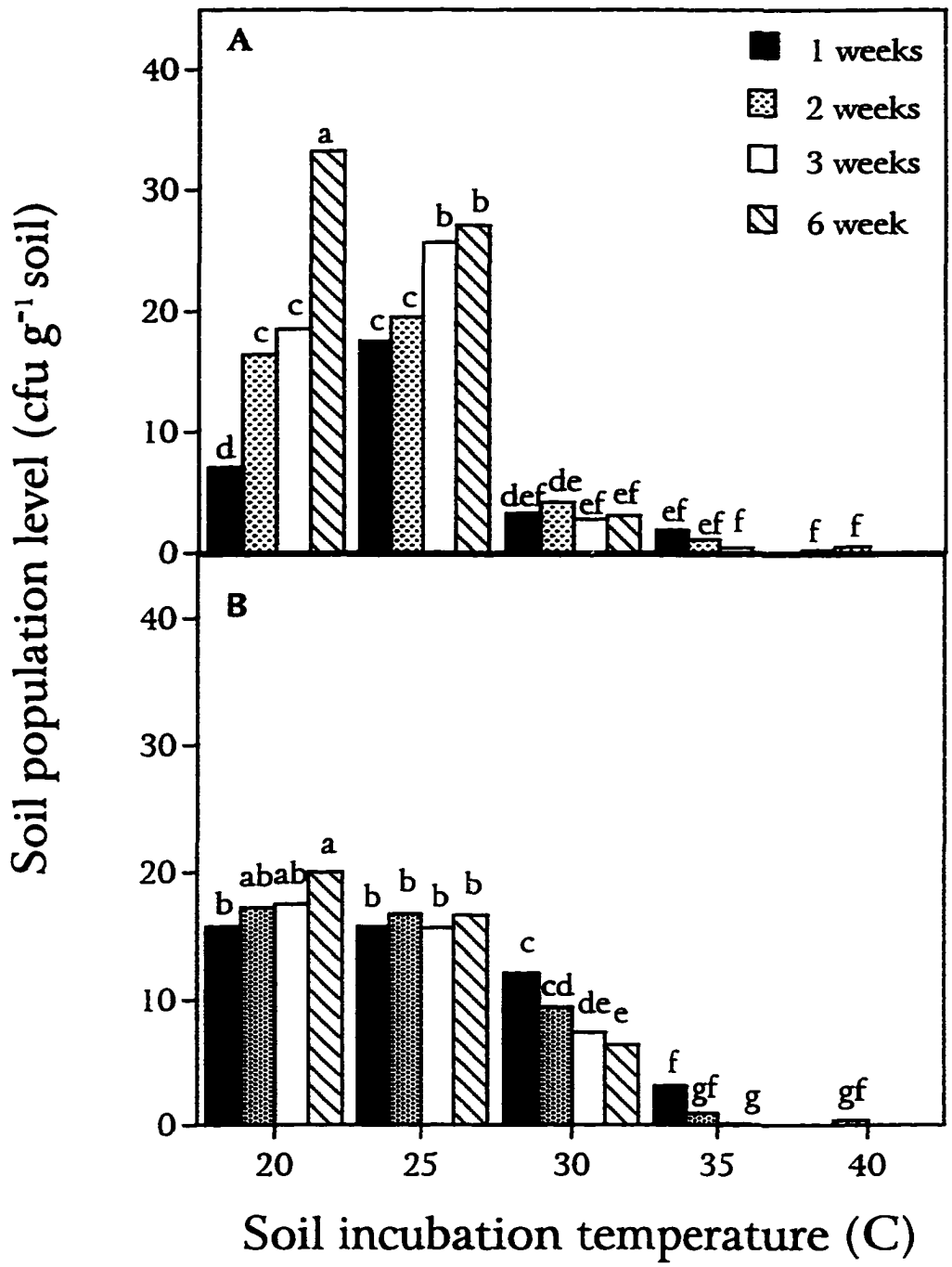

Fig.3.3. Bars represent population levels of *Calonectria ilicico;a* after incubation of infested soil for 1, 2, 3, or 6 weeks at 20, 25, 30, 35 or 40°C (A) and when soybeans grown in these soil were harvested after 8 weeks (B). Treatment means marked with the same letter are not significantly different within each panel according to least squares means ($P \leq 0.05$).

Although there were differences depending on incubation temperatures and durations, root colonization levels in soybeans grown in infested soil previously exposed to 20, 25, and 30°C were significantly higher (between 28% and 45 %) than those grown in soil exposed to 35 and 40°C (between 8% and 0%) (Fig. 3.4). Root colonization was negligible in soybeans grown in soil previously exposed to 40°C (Fig. 3.4).

Effect of soil temperature on root colonization. In each of these root systems single *C. ilicicola* colony was produced mainly closer to the crown region. The fungus was not recovered from lateral roots. Soybean plants grown at 20, 25, 30, and 35°C daytime soil temperatures were infected, but not soybeans grown at 40°C. The number of infected plants was higher at 25, 30, and 35°C daytime soil temperatures than at 20 and 40°C.

Effects of temperature and infestation density were significant on soybean tap and lateral root colonization. Taproot colonization increased as day temperatures increased from 20 to 30°C but was significantly decreased at higher temperatures (Fig. 3.5). At a daytime soil temperature of 40°C, taproot colonization was detected only in the highest soil infestation level (Fig. 3.5). Lateral root colonization increased significantly as the daytime soil temperature increased from 20 to 30°C, and the maximum lateral root colonization was detected at 30°C (Fig. 3.6). Lateral root colonization decreased significantly with further increase in daytime temperature (Fig. 3.6). Lateral root colonization levels were lowest at 20, 35, and 40°C (Fig. 3.6).

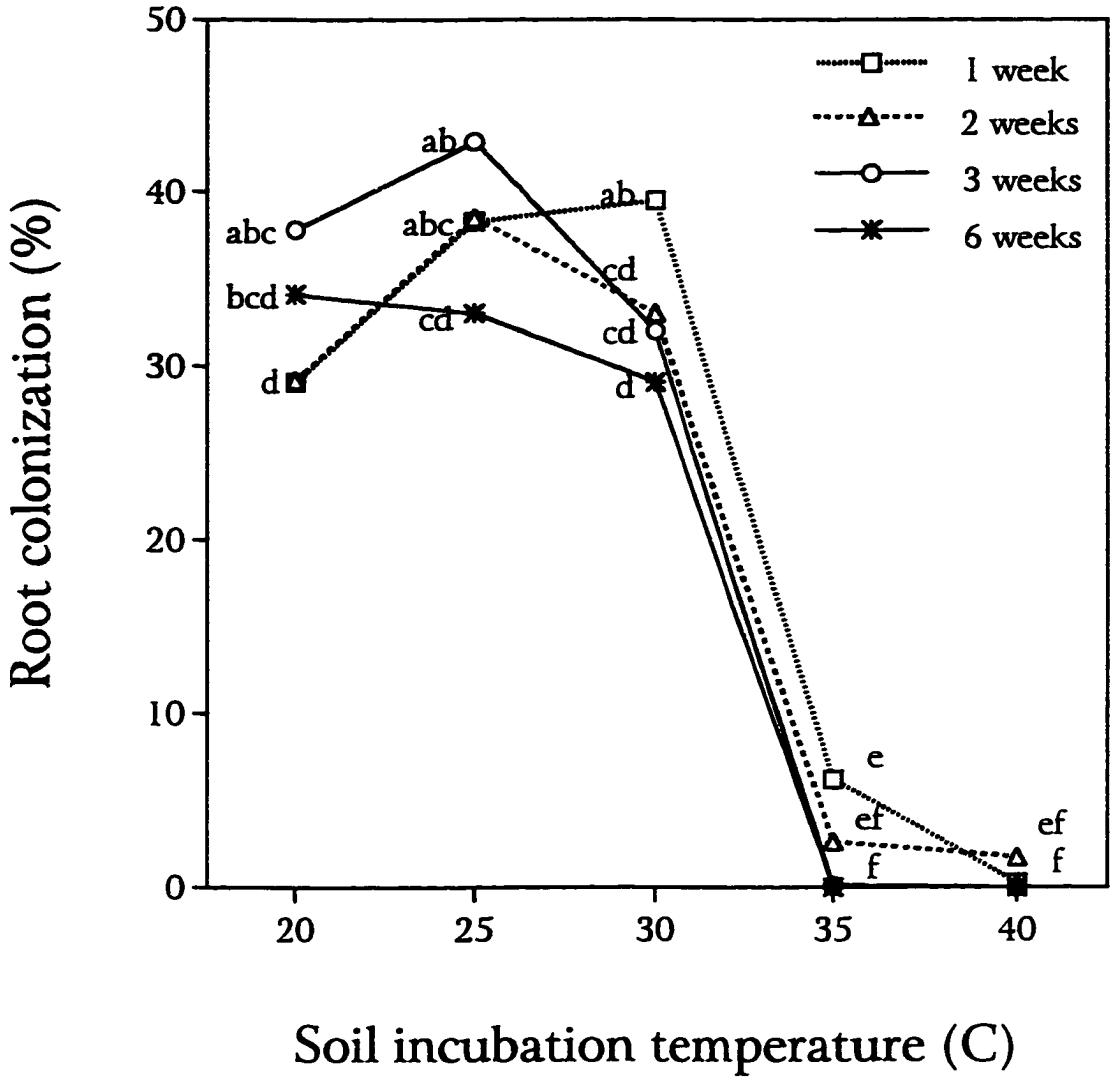

Fig.3.4. Root colonization by *Calonectria ilicicola* in soybeans grown at 25°C for 8 weeks in a green house in soil infested with microsclerotia. The soils were previously incubated at 20, 25, 30, 35 or 40°C for 1, 2, 3, or 6 weeks. Treatment means marked with the same letter did not differ significantly according to least squares means ($P \leq 0.05$).

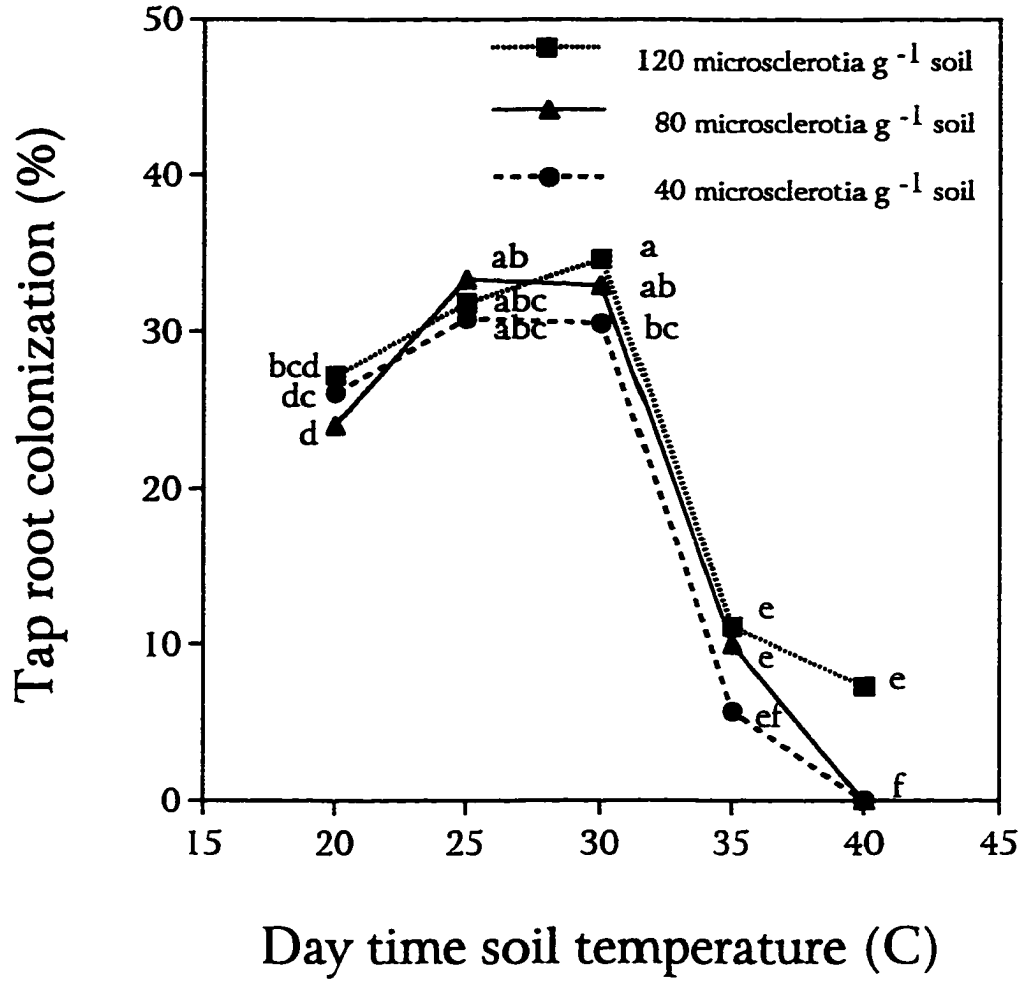

Fig. 3.5. Taproot colonization of soybean by *Calonectria ilicicola*. Soybean plants were grown in 3 soil inoculum densities at 20, 25, 30, 35, and 40°C daytime soil temperatures (15, 20, 25, 30, and 35°C nighttime soil temperatures, respectively). Treatment means marked with the same letter did not differ significantly according to least squares means ($P \leq 0.05$).

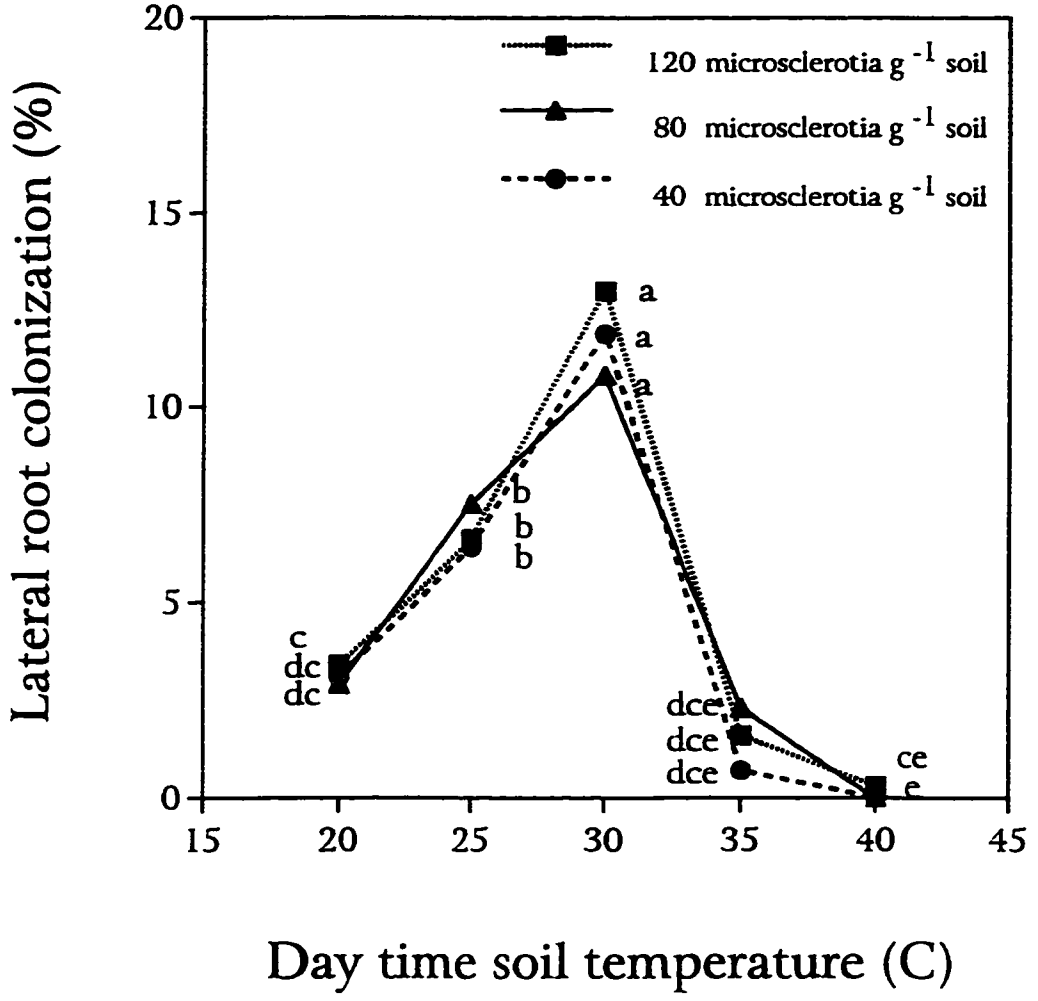

Fig.3.6. Lateral root colonization of soybean by *Calonectria ilicicola*. Soybean plants were grown in 3 soil inoculum densities at 20, 25, 30, 35, and 40°C daytime soil temperatures (15, 20, 30, and 35°C nighttime soil temperatures, respectively). Treatment means marked with the same letter did not differ significantly according to least squares means ($P \leq 0.05$).

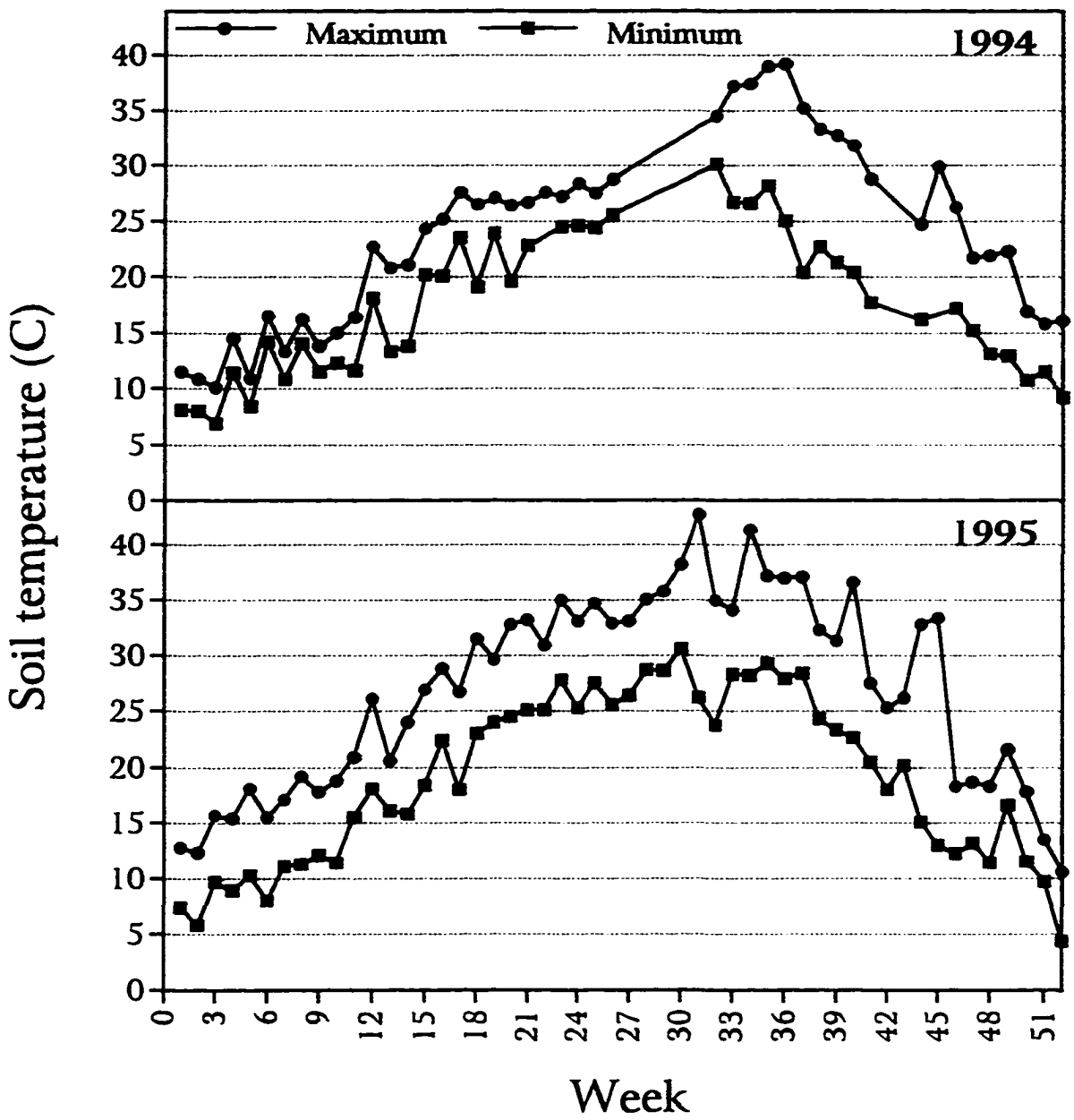

Fig.3.7. Maximum and minimum weekly average soil temperatures at 10 cm depth in soybean experimental fields at Ben Hur Research Farm, Baton Rouge, LA in 1994, and 1995.

During 1994, weekly average soil maximum temperatures in a soybean field at a depth of 10 cm were 25-30^{0}C during the optimal planting time (week 21) through week 26 (Fig. 3.7). The soil temperature had increased to more than 30^{0}C by week 32 and was 35-39^{0}C for a 5 wk period beginning week 33. The weekly average soil maximum temperature decreased to $\leq$ 30^{0}C over the next 4 wks (Fig. 3.7). In 1995, weekly average soil maximum temperatures during optimal soybean planting time (week 19-21) were in 30-35^{0}C range and remained at this level for the rest of the growing season (until week 42) (Fig. 3.7).

Discussion

Early root colonization by *C. ilicicola* is critical for the development of red crown rot disease in soybean (Kuruppu and Russin, 1996). Our previous work showed that there is a positive relationship between the *C. ilicicola* soil population and soybean root colonization (Chapter II). Therefore, the initial pathogen inoculum level in soil may be an important factor in red crown rot development in soybean. Reduction of initial soil inoculum level through cultural and chemical methods is recommended for black root rot management in peanut (Sidebottom and Beute, 1989; Phipps, 1990). According to published reports, temperature is the most important environmental factor affecting *C. ilicicola* population level in peanut soils (Phipps and Beute, 1977; Griffin *et al.*, 1978, Pataky and Beute, 1983). However, studies of soil temperature effects on the survival of *C. ilicicola* in peanut soils were focused on low temperature limits (Roth *et al.*, 1979; Griffin *et al.*, 1978) and extreme winter temperature effects (Taylor *et al.*, 1981).

High temperature effects on the survival structure of several soil borne pathogens were studied in some detail (Bega and Smith, 1962; Sheikh and Ghaffar, 1986; Nelson and Weilhelm, 1958; Subbarao and Hubbard, 1996). However, high temperature effects on *C. ilicicola* microsclerotia have hardly been investigated. In our study the main focus was on the upper soil temperature limits of this pathogen. Results of the current study show that high soil temperatures during summer play a critical role in controlling the inoculum level of this fungus in heavy alluvial soils in soybean fields in Louisiana. According to these results, the optimal soil temperature range for the survival of *C. ilicicola* microsclerotia in heavy alluvial soil is 20-30^{0}C and the maximum soil temperature limit is 35^{0}C. However, the optimal soil temperature range for germinability of microsclerotia is only 20-25^{0}C. Results of these experiments also show that the longer the soil temperature is in the optimal range for germinability, the higher will be the level of germinable microsclerotia in soil. When the soil was at a marginal temperature (30^{0}C) level, the number of germinable microsclerotia was reduced considerably. The optimal soil temperature range for the maintenance of infectivity of *C. ilicicola* microsclerotia (i.e., their ability to germinate in the rhizosphere and infect soybean roots) is 20-30^{0}C. Although the germinable microsclerotia number was considerably reduced when infested soil was exposed to 30^{0}C for 1 to 6 wks, root colonization levels in soybeans grown in this soil were as high as those grown in soil incubated at 20 or 25^{0}C (the optimal temperature range for germinability). Germinable microsclerotia number was also higher by the time soybeans were harvested than that was

detected immediately after incubation at 30^{0}C (Fig. 3.5B). These results suggest that the loss of germinability caused by exposing soil to 30^{0}C could have been a temporary effect in some of these microsclerotia, and that germinability could be regained if microsclerotia were exposed to a lower temperature for several weeks. A temporary loss of microsclerotia germinability caused by low temperatures (-10^{0}C or 6^{0}C for 4 wks) was observed by Roth *et al.* (1979). The low temperature effect was partially alleviated when soil incubated at low temperatures was transferred back to 25^{0}C for 4 wks, which suggested that low temperature does not always cause a permanent loss of microsclerotia viability in *C. ilicicola*. Our results show that the soil temperatures above 35^{0}C affected not only germinability but also infectivity of these microsclerotia. Exposure to temperatures >35^{0}C for a period as short as 1 wk may considerably reduce the number of viable microsclerotia in soil. Therefore, 35^{0}C can be considered as the high temperature limit for the survival of *C. ilicicola* microsclerotia. Because the number of germinable microsclerotia may change depending on the soil temperature and duration exposed to that temperature prior to sampling, the value may not always reflects the true inoculum level of this fungus in soil.

Recoverable levels of *C. ilicicola* microsclerotia were reduced considerably when incubated in soil at all the temperature levels used in the current study. Loss of germinability of sclerotia of *Sclerotium rolfsii* (Hyakumachi and Lockwood, 1989) and *Macrophomina phaseolina* (Papavizas, 1977) during incubation in soil also has been reported. Reduction of germinability and pathogenic aggressiveness during incubation in soil of

some of these fungal propagules has been attributed to the lose of their endogenous nutrients due to respiration and diffusive stress imposed by other soil microorganisms competing for carbon substrates (Sneh and Lockwood, 1976; Hyakumachi and Lockwood, 1989; Mondal *et al.*, 1995). In the current study, although the germinable microsclerotia number always decreased when incubated in soil, this number increased as the incubation duration increased from 1 wk through 6 wks when incubated at 20^{0}C and from 2 wks to 3 and 6 wks when incubated at 25^{0}C. It may be possible that the temporary quiescent state imposed by the exposure to soil on these microsclerotia was alleviated with time at temperatures favorable for the fungus.

Microsclerotia used in this study were produced on nutrient media in the laboratory. Linderman and Gilbert (1972) suggested that sclerotia of *Sclerotium rolfsi* produced in cultures may structurally and physiologically different from those produced in soil. Laboratory produced microsclerotia of *C. ilicicola* may behave differently from microsclerotia produced in the field. Therefore, interpretation of the behavior of microsclerotia in the field based on the information obtained from laboratory produced microsclerotia may not be accurate but still might provide useful information on the behavior of the pathogen under field conditions. Based on findings of thr current study we are able to explain the inconsistencies in *C. ilicicola* population in soybean delayed planting field experiments conducted at Louisiana State University Agriculture Center Ben Hur Research Farm in 1994, 1995 and 1996 (Chapter II). We have detected an increase in the level of germinable microsclerotia in the field soil (from 2

to 17 microsclerotia g-1 soil) and considerable level of soybean root colonization during summer 1994 (Chapter II). The reason may be that the soil temperature was in the optimal temperature range for germinability and infectivity of *C. ilicicola* microsclerotia for several weeks prior to and during optimal planting time (Fig. 3.7). In 1995, soil temperatures exceeded the optimal range for germinability (> 30^{0}C) during late spring (3 wks prior to optimal planting) and reached the maximum limit for the pathogen survival (35^{0}C) during early summer (Fig. 3.7) which coincided with a dramatic decrease in the number of germinable microsclerotia in this field (Chapter II). Soil temperature level was above the high temperature limit for this pathogen for nearly 7 wks and even reached >40^{0}C during a period of 2 wks (Fig. 3.7). This reduced level of viable microsclerotia in soil for the remainder of 1995 and in 1996 in this field, was consequently leading to comparatively low level of soybean root colonization during the growing seasons in these 2 years (Chapter II).

Soil temperature was reported to be the most important environmental factor affecting the infection of peanut roots by *C. ilicicola* (Bell, 1966; Phipps and Beute, 1977). During the current study, we examined the optimal soil temperature range favorable for root colonization by *C. ilicicola* in soybeans grown in heavy alluvial soil in Louisiana. Our findings indicated that *C. ilicicola* invaded seedling roots when soybeans were grown at soil temperatures ranging from 25 to 35^{0}C. However, the optimal soil temperature range for taproot colonization of young soybean plants by the red crown rot fungus was 25-30^{0}C whereas for lateral root colonization it was 30^{0}C. Soybean root colonization level by *C.*

ilicicola in this soil temperature range was independent of soil pathogen density at the soil pathogen densities tested. Taylor *et al.* (1981) described a linear relationship of field soil population and peanut root colonization, but the inoculum density range described in his study was only 0-14 microsclerotia g^{-1} soil. Even though *C. ilicicola* could invade soybean seedlings, root colonization and probably further root invasions were suppressed when the soil temperature was 35^{0}C. This fungus was not able to invade and/or colonize soybean roots at 40^{0}C when soil population density was low. However, the fact that some level of root colonization was detected at 40^{0}C at the highest population density indicates that a few microsclerotia in soil can infect at this soil temperature if the soil is heavily infested.

An examination of average soil temperature during summer in Louisiana soybean fields shows a short period (i.e., a few weeks) during early summer when soil temperature levels are conducive (20-30^{0}C) for soybean root colonization by *C. ilicicola*. Our previous work suggested the importance of early soybean root colonization for the later development of red crown rot symptoms (Kuruppu and Russin, 1996). If soybeans are planted during the conducive period, there will be a considerable level of early root colonization consequently resulting in the development of red crown rot symptoms. Based on the findings of the current study, high soil temperatures control the *C. ilicicola* survival in field soil as well as the soybean root colonization by this fungus. Therefore, soil temperature can be considered as the most important environmental factor controlling the development of red crown rot in soybeans in Louisiana.

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CHAPTER 4

ROOT COLONIZATION OF SOYBEAN BY *CALONECTRIA ILICICOLA*, THE RED CROWN ROT FUNGUS AS INFLUENCED BY HOST AGE

Introduction

Red crown rot of soybean, *Glycine max* (L.) Merrill is caused by the soilborne fungus, *Calonectria ilicicola* Boedijn & Reitsma (Rowe *et al.*, 1973; Berggren and Snow, 1989; Crouse *et al.*, 1993) (synonym *Calonectria crotalariae* (Bell and Sobers, 1966; Crouse *et al.*, 1993). *Cylindrocladium parasiticum* (synonym *Cylindrocladium crotalariae*) is the imperfect stage of this fungus (Bell and Sobers, 1966; Crouse *et al.*, 1993). Foliar symptoms of red crown rot usually appear during beginning pod (R_3) to full pod (R_4) (Fehr *et al.*, 1971) soybean growth stages and include leaf chlorosis and interveinal necrosis followed by defoliation (Berggren and Snow, 1989). The fungus colonizes soybean roots during early vegetative stages (Chapter II) and diagnostic reddish-brown perithecia appear in the crown region coincidentally with leaf symptoms. In the United States, this disease was first reported in North Carolina in 1973 (Rowe *et al.*, 1973) and was first reported in Louisiana in 1976 (Berner *et al.*, 1986). In 1966, Bell and Sobers described this fungus as the causal agent for black root rot, also known as Cylindrocladium black rot, of peanut (*Arachis hypogea*).

Delays in planting reduce incidence of soybean red crown rot (Russin *et al.*, 1985; Berner *et al.*, 1988). Recommended disease management strategies in Louisiana include delayed planting and use of less susceptible cultivars (Berner *et al.*, 1988; Berggren and Snow, 1989). However delayed planting is the management strategy of choice for red crown rot because of lack of resistant cultivars. The mechanism behind reduced disease incidence following delayed planting is not clear.

According to Kim *et al.* (1998), younger soybean plants (< 20 days old) were less susceptible to *C. ilicicola* than plants 20-30 days old. Low susceptibility of young plants to the pathogen would reduce initial root colonization if soybeans were planted in the period during which field conditions are most conducive for root colonization. Because Kim *et al.* (1998) used a non-representative inoculation method, it is not known whether his results actually depict root colonization in the field. It is important to examine the susceptibility of soybean plants to *C. ilicicola* during the early growth stages because early root colonization is critical for the late development of red crown rot in soybeans (Kuruppu and Russin, 1996). Therefore, the objective of this study was to examine effects of host age on soybean root colonization by *C. ilicicola*.

Materials and Methods

Soil for these experiments was collected from the Louisiana State University Agriculture Center Ben Hur Research Farm, Baton Rouge. The soil type was Mhoon silty clay loam (in order Inceptisols, suborder: Aquept, in subgroup: Fluventic Haplaquepts, in family: Fine-silty, mixed nonacidic, thermic) and was free of *C. ilicicola*.

Inoculum preparation. *Calonectria ilicicola* isolate SG915 (Kim, 1994) was used to infest soil. Fungus cultures were grown on potato dextrose agar (PDA) at 25^{0}C for 6 wks. Mycelia in agar were blended in distilled water for 1 min and the slurry was poured through nested sieves of 425 μ pore size over 150 μ pore size. Material on the 150 μ pore size sieve was washed under a stream of water to dislodge and remove hyphal fragments. Microsclerotia were then suspended in distilled water and enumerated.

Host age effects on root colonization. Experiments were conducted in a greenhouse. Pots (20 cm in diameter) were filled with 1 kg of field soil free of *C. ilicicola*. In each pot, 8 glass test tubes (1 x 8 cm) were placed to facilitate soil infestation without damaging plant roots. Glass tubes were placed in 2 circles (4 on each circle) in soil. Positions of tubes in the outer circle alternated with those in the inner circle. Tubes in the outer circle were forced 8 cm deep into soil and 4 glass tubes on the inner circle were forced 4 cm deep into soil. A single pregerminated seed of soybean cultivar Sharkey was planted at the center of 12-cm-diameter plastic pots. In order to get 6 soybean plants of each age group, 6 soybean seeds were planted each week, and planting was continued for 9 wks. On the ninth week, glass tubes were carefully removed and 100 ml of microsclerotia suspension sufficient concentration to give 50 microsclerotia g^{-1} soil (prepared as described previously) was pipetted into all holes in each pot. Equal volumes of microsclerotia suspension were added to each hole. Holes were filled with dry soil while the microsclerotia suspension was being added. Ninth batch of seeds was planted in soil infested with microsclerotia. Plants were allowed to grow in the greenhouse (25 ± 5^{0}C) and were watered once each day. Plants were harvested after 2 wks.

Determination of tap and lateral root colonization. Soybean roots were freed from soil by gentle washing under a stream of water. Tap and lateral roots were separated and then cut into segments 1 cm in length. From these, 20 taproot and 50 lateral root segments were selected at random. These root segments were surface sterilized in 0.25% NaOCl for 30 sec, rinsed 3 times in sterile water, and blotted on sterile filter paper. These

root segments were plated on modified Phipps medium (Phipps *et al.*, 1976) and incubated at room temperature (25-27^{0}C) under continuous fluorescent light for 10-14 days. Root colonization was expressed as percentage of segments from which colonies of *C. ilicicola* were recovered. Data were analyzed using SAS General Linear Models procedure (SAS Institute, Cary, NC) to determine the host age effect on soybean root colonization by *C. ilicicola*.

Results

The effect of host age at the time of pathogen infestation was significant for both tap and lateral root colonization. Maximum taproot (50%) as well as lateral root (35%) colonization was detected when soybean seeds were planted in infested soil (Fig. 4.1). Both tap and lateral root colonization levels were reduced approximately 50% when the pathogen was introduced to 1-week-old plants (Fig. 4.1). Root colonization remained more or less at this level until the seventh week after seedling emergence. Then there was an increase in root colonization during the eighth week. However, to examine whether there is an increasing trend in susceptibility of soybean plants to *C. ilicicola* after this age, the experiment would have continued for several more weeks.

Discussion

Several investigators (Russin *et al.*, 1985; Berner *et al.*, 1986) reported that red crown rot incidence in soybean was reduced by delaying planting. Previously we examined how soybean root colonization by *C. ilicicola* was affected by delays in planting (Chapter II). Initiation of root colonization

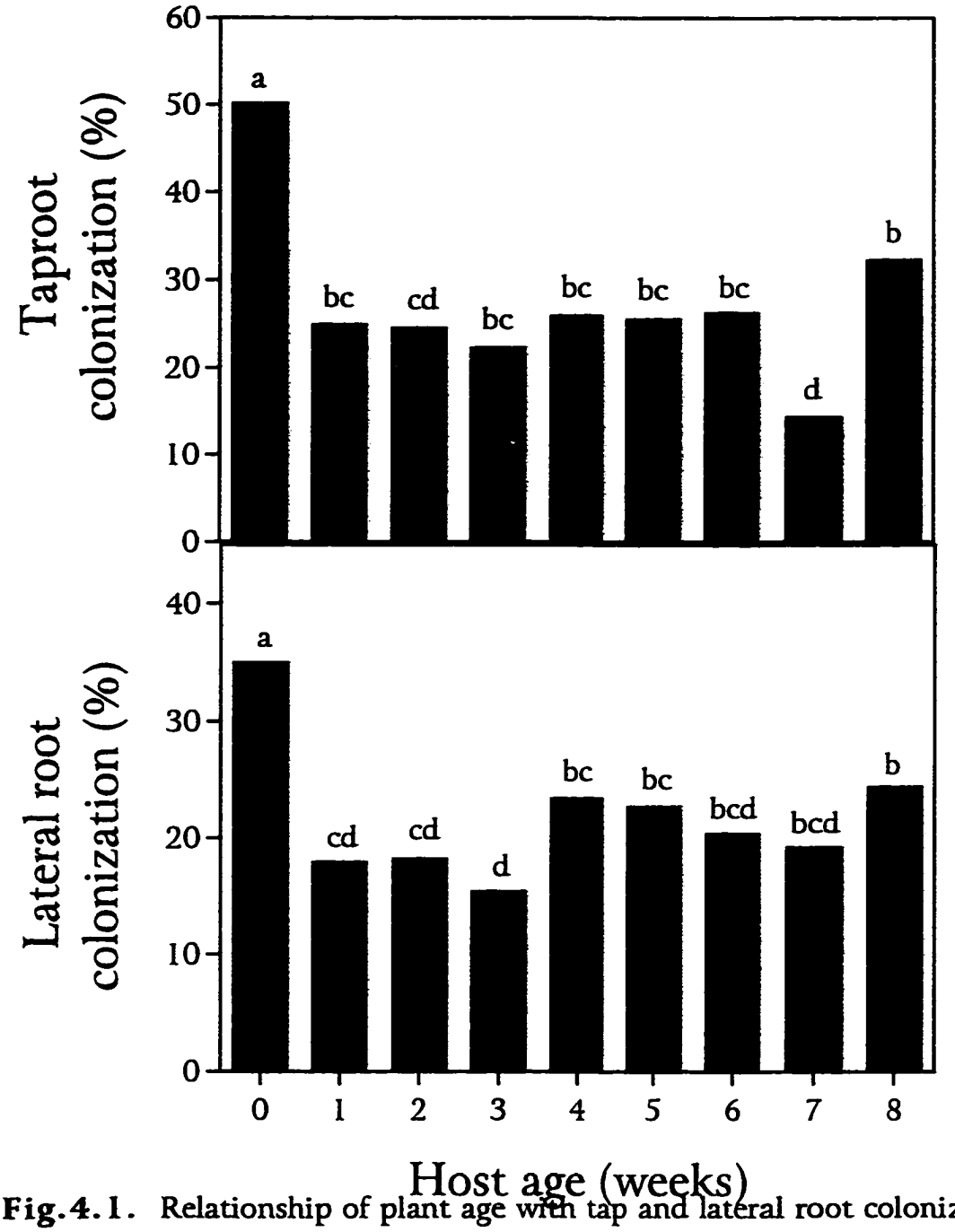

Fig. 4.1. Relationship of plant age with tap and lateral root colonization of soybeans by *Calonectria ilicicola*. Soil was infested with *C. ilicicola* microsclerotia when soybean plants were 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks old and root colonization was determined 2 week later. Bars with same letter in each panel did not differ significantly according to least squares means ($P \leq 0.05$).

was delayed and root colonization levels were reduced when susceptible soybean cultivars were planted 3 or 6 wks later than the optimal time.

Differential host responses to pathogens based on host age have been reported for several crops. Soybean plants in early growth stages (Fehr *et al.*, 1971) were more susceptible to stem canker caused by *Diaporthe phaseolorum* var. *caulivora* (Smith, 1989; Padgett, 1992) but less susceptible to brown stem rot caused by *Phialophora gregata* (Phillips, 1972). Kim *et al.* (1998) reported that younger soybean plants were less susceptible to infection of *C. ilicicola*, which is opposite of the findings of our study. They suggested that the reduced disease incidence following delayed planting may result from reduced susceptibility of young plants. In their experiments, however soybean plants of different ages were inoculated by placing mycelium of *C. ilicicola* in agar disks at stem bases and covering them with soil. Their conclusions were based on length of these lesions and number of perithecia produced on stems. We consider this is a non-representative method of inoculation; a method closer to the natural system of root invasion by the pathogen in the field is needed to make any inferences on the field situation. In our experiments, we exposed different vegetative growth stages of soybean plants to the pathogen by infesting field soil in pots with *C. ilicicola* microsclerotia produced in the laboratory. We were careful not to damage soybean roots during the infestation process. In the current study, up to 9-week-old soybean plants were examined, because according to our field studies the critical period of root colonization for red crown rot symptom development was for about 7 wks after planting. Contrary to findings of Kim *et al.* (1998), our results showed that soybean plants were most susceptible to *C. ilicicola* during the first

week after seedling emergence. Host susceptibility was reduced during the next few weeks at least till the eighth week after seedling emergence.

These results show that availability of infective inoculum in soil and optimal environmental conditions conducive for root colonization provide maximum opportunity for soybean root colonization by *C. ilicicola* during the first week after seedling emergence. Previously we have shown that, if soil temperature in a soybean field infested with *C. ilicicola* was in the range of 20 – 25^{0}C for several weeks prior to planting, there could be a considerable amount of infective microsclerotia in soil (Chapter III). According to our previous work this temperature range is the optimal range for soybean root colonization by this pathogen as well (Chapter III). This soil temperature range prevails during the optimal soybean planting time in Louisiana. If soybeans are planted during the optimal soybean planting time, the most susceptible stage of the host is exposed to high level of infective microsclerotia in soil as well as the soil temperature conditions conducive for soybean root colonization by *C. ilicicola*. Roots of those soybeans planted during the optimal time would be considerably colonized during the early season which is critical for the later development of red crown rot.

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CHAPTER 5

SUMMARY

This study was designed to examine some of the important biological and epidemiological aspects of red crown rot disease development in soybean. Soybean root colonization by *C. ilicicola* and subsequent red crown rot disease incidence, as well as, changes of *C. ilicicola* population in a soybean field, were examined over three years. Results revealed that soybean roots are colonized by *C. ilicicola* during early vegetative growth stages, but do not necessarily exhibit root discoloration or necrosis. According to our results, early soybean root colonization (about seven weeks after planting) by *C. ilicicola* is critically important for development of red crown rot symptoms in late reproductive stages. Early colonization of soybean taproots as well as lateral roots by *C. ilicicola* was important for red crown rot symptom development.

Initiation of soybean root colonization was delayed and root colonization levels were reduced in the more-susceptible cultivar Sharkey following delayed planting, resulting in low red crown rot incidence. Because field conditions are optimal for soybean root colonization only for a short period early in summer, it is advisable to avoid this period when planting susceptible soybean cultivars in fields with a history of red crown rot.

Root colonization during the critical period, as well as disease incidence, both were low in the less-susceptible cultivar Cajun, regardless of planting date. This indicates the effectiveness of planting less-susceptible cultivars for soybean red crown rot management and stresses the importance of developing resistant soybean cultivars. A possible relationship between reduced disease in the less-susceptible cultivar and its ability to limit early lateral root colonization by *C. ilicicola* was identified.

A positive correlation between soybean root colonization and *C. ilicicola* population levels in field soil was detected. The correlation between taproot colonization and soil population level was weak but positive in all three cropping seasons (1994, 1995, and 1996). A strong positive correlation between lateral root colonization and soil population was detected only in 1994, the only year in which red crown rot symptoms were expressed in soybean plants. This was likely due to deterioration of heavily colonized lateral roots (towards the end of the year), which contributed to the increase in *C. ilicicola* population in field soil. Negligible levels of early season root colonization in field plots in 1995, in spite of high soil pathogen population, suggested a probable involvement of high temperature experienced during planting time in reducing root colonization by *C. ilicicola*. Soil population levels under soybean cultivars differing in susceptibility were not consistent during 1994, 1995, and 1996 in this field. A higher *C. ilicicola* population level under more-susceptible Sharkey was detected towards the end of 1994 but the difference disappeared after spring 1995. During the early summer 1995, *C. ilicicola* population level in this field soil decreased dramatically and continued to be low for the remainder of the year as well as throughout 1996 growing season, even in the presence of a susceptible host. We found that microsclerotia of *C. ilicicola* are affected by unusually high temperatures experienced during growing seasons of 1995 and 1996.

High temperature effects on *C. ilicicola* microsclerotia in a heavy alluvial soil common to Louisiana soybean fields were examined in a series of laboratory and greenhouse experiments. Field soil infested with laboratory-produced microsclerotia was exposed for different durations to a

range of temperatures experienced during the soybean growing season in Louisiana. The ability of microsclerotia to germinate and produce colonies on nutrient media after recovering from soil was referred to as germinability. Microsclerotia germinability was affected by soil temperature, as well as, the duration of exposure to that temperature. Optimal soil temperature range for germinability of these microsclerotia was 20-25^{0}C. The longer the soil temperature was in the optimal range for germinability, the higher was the level of germinable microsclerotia in soil. This level was considerably reduced with longer exposure to 30^{0}C, which appeared to be a marginal soil temperature for microsclerotia germinability. According to these results, the number of microsclerotia recovered from field soil in laboratory may not always reflect the real field *C. ilicicola* population level, as microsclerotia germinability may be influenced by soil temperatures at and prior to soil sampling. The ability of microsclerotia to germinate in the soybean root rhizosphere and invade soybean roots was defined as infectivity of microsclerotia. The optimal soil temperature range for infectivity of *C. ilicicola* microsclerotia was 20-30^{0}C. These microsclerotia did not survive at soil temperatures $\geq$ 35^{0}C. Changes of *C. ilicicola* population levels observed in experimental field plots in 1994, 1995, and growing season in 1996 are explained by these findings. Results of this study indicate that the number of infective and viable *C. ilicicola* microsclerotia in soybean fields decrease in response to increasing soil temperatures during the growing season.

Experiments conducted in growth chambers examined the soil temperature effects on soybean root colonization by *C. ilicicola*. The

optimal temperature range for soybean taproot colonization was 20-30^{0}C and for lateral root colonization was 25-30^{0}C. Although *C. ilicicola* can invade soybean roots at 35^{0}C, this temperature was not favorable for root colonization. These results support the role for high soil temperature for reduced root colonization during optimal planting time in 1995. In 1994, the only year in which red crown rot disease symptoms were expressed in soybeans, soil temperatures were optimal for soybean root colonization from April until the end of June.

Susceptibility to *C. ilicicola* at different soybean plant ages was examined in greenhouse experiments. The first week after seedling emergence was the most susceptible period of the soybean plant for *C. ilicicola* infection. Host susceptibility was reduced during the next few weeks at least till the eighth week after seedling emergence. Therefore, the highest soybean root invasion and colonization by *C. ilicicola* can be expected during the first week after seedling emergence, if field conditions are favorable during that time.

Based on our results, we are able to explain the high levels of red crown rot incidence in soybeans following optimal planting. When planted during the optimal time, the most susceptible plant stage is exposed to infective microsclerotia in soil at temperatures generally conducive for root colonization. The critical early root colonization period may overlap with the most favorable soil temperature conditions for root colonization. During this time, infective microsclerotia in soil cause substantial levels of early root colonization which subsequently result in high levels of disease incidence. However, the level of infective microsclerotia in field soil will

gradually decrease in response to increasing soil temperatures as the growing season progress. The soil temperature level might exceed the optimal range for soybean root colonization as well. Delaying soybean planting by a few weeks may expose the most susceptible host stage to a lower number of infective microsclerotia at temperatures that are less conducive for root colonization. During the critical period for root colonization, conditions may be less favorable than that following optimal planting. This explains how early soybean root colonization is reduced in susceptible soybean cultivars planted a few weeks late.

Areas for Future Research

We examined red crown rot disease development in soybean for three years and attempted to understand several biological and epidemiological aspects of this disease. There were some questions raised during the study. Addressing these questions will help understand the soybean-*Calonectria ilicicola* pathosystem.

1. Based on our results, colonization of soybean lateral roots was more closely associated with red crown rot symptom development than was that of taproots. This may be an important aspect to be examined in detail.
2. Hardly any information is available on root infection process by *C. ilicicola* and subsequent pathogen development within the soybean root system. Histological studies of soybean root colonization by *C. ilicicola* will provide basic information on this aspect.
3. The current study stresses the importance of planting resistant cultivars in the management of red crown rot in soybean. Although red crown rot resistant cultivars are not available, some soybean cultivars are known to be less susceptible. Understanding the mechanism behind this

reduced susceptibility will provide information useful to enhance breeding and selection of soybean cultivars resistant to red crown rot.

4. During the current study, there were indications that low rainfall has an effect on *C. ilicicola* microsclerotia survival and on soybean root infection by *C. ilicicola* as well. Effects of soil moisture, as well as the interaction of soil moisture with soil temperature, on *C. ilicicola* microsclerotia and on soybean root infection by this fungus need to be examined.

APPENDIX

DOES AGE OF *CALONECTRIA ILICICOLA* MICROSCLEROTIA AFFECT SENSITIVITY TO SOIL TEMPERATURE ?

Materials and Methods

The effects of temperature on the germinability and infectivity of *C. ilicicola* microsclerotia were tested in a greenhouse experiment. Field soil was infested with microsclerotia of *C. ilicicola* isolate SG 915 that were produced in the laboratory on potato dextrose agar (PDA). Fungus cultures were grown on PDA at 25^{0}C for 8 wks. Microsclerotia suspension was prepared as described in Chapter III. One kilogram of soil in each plastic bag was infested with *C. ilicicola* microsclerotia using the procedure described in Chapter III. The amount of water that should be added to bring this soil to field capacity was predetermined and added to soil in plastic bags and then the weight of each bag was recorded. These bags of soil were incubated at 20, 25, 30, 35, and 40^{0}C for 3 or 6 wks. Soil moisture level was maintained at field capacity during incubation by adding enough water to each bag to bring back to its initial weight. Each temperature treatment was replicated 5 times. At the end of each incubation period the number of germinable microsclerotia in soils incubated at each temperature was assayed using the method described in Chapter III.

To determine the infectivity of microsclerotia exposed to these different soil temperatures, seeds of soybean cultivar Sharkey were planted into 12-cm-diameter plastic pots (1 seed per pot) containing incubated soil. Each treatment was replicated 5 times. Plants were allowed to grow in a greenhouse at 25$\pm$5^{0}C and plants were harvested after 8 wks. Lateral and taproot colonization levels were assayed separately using the

method described in Chapter III. Soil population levels were assayed again at the end of the experiment. This experiment was conducted only one time. Data were analyzed using SAS General Linear Models procedure [SAS Institute, Cary, NC] to determine the main and interactive effects of temperature and duration on germinability and infectivity of *C. ilicicola*.

Results

Calonectria ilicicola microsclerotia were sensitive to incubation temperatures as well as duration of incubation at that temperature. When averaged across 2 incubation durations, microsclerotia germinability decreased as incubation temperature increased. Very few microsclerotia germinated when incubated at temperatures above 35^{0}C and that number was negligible at 40^{0}C (Fig. A1). More microsclerotia were germinable after 6 wks incubation than at 3 wks incubation when incubation temperatures were 20 or 25^{0}C (Fig. A1). Incubation duration did not have an effect on the number of germinable microsclerotia at 30^{0}C (Fig. A1). Levels of germinable micorsclerotia in soil were considerably lower by the time soybeans were harvested than those immediately following incubations at different temperatures. At that time microsclerotia levels were different only in infested soil incubated at 20^{0}C (Fig. A1).

Highest root colonization level was detected in soybeans grown in infested soil incubated at 25^{0}C (Fig. A2). Higher level of root colonization was detected in soybeans grown in infested soil incubated for 3 wks than 6 wks at 20^{0}C (Fig. A2). Root colonization levels were not affected by the duration when infested soil was incubated at 30^{0}C and this level was similar to that in infested soil incubated at 20^{0}C for 6 wks (Fig. A2). Only

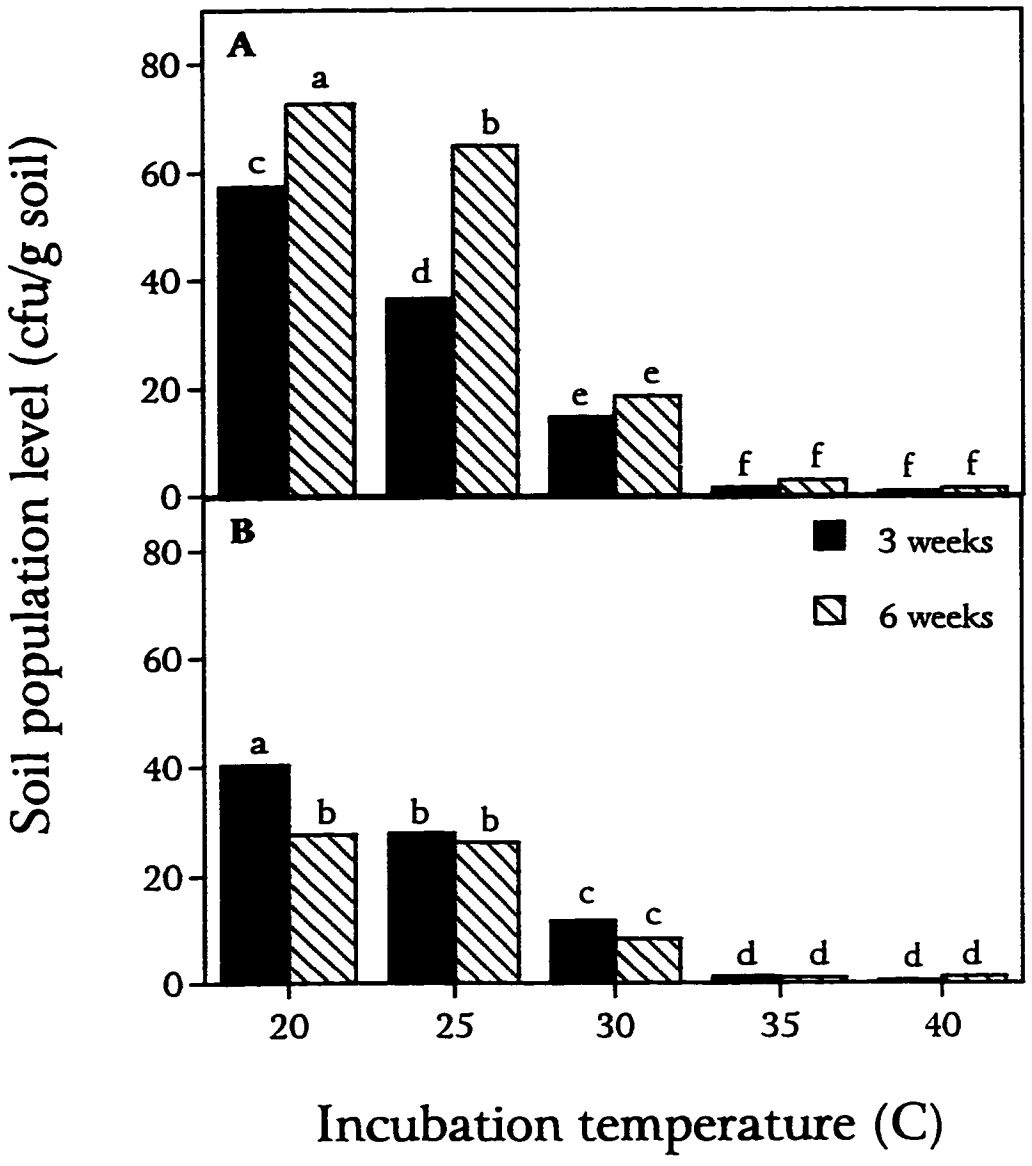

Fig.A1. Levels of germinable microsclerotia of *Calonectria ilicicola* after incubating in soil for 3 and 6 weeks at 20, 25, 30, 35, and 40°C (A) and when soybeans grown in these soil were harvested 8 weeks later (B). Treatment means marked with the same letter are not significantly different within each panel according to least squares means ($P \leq 0.05$).

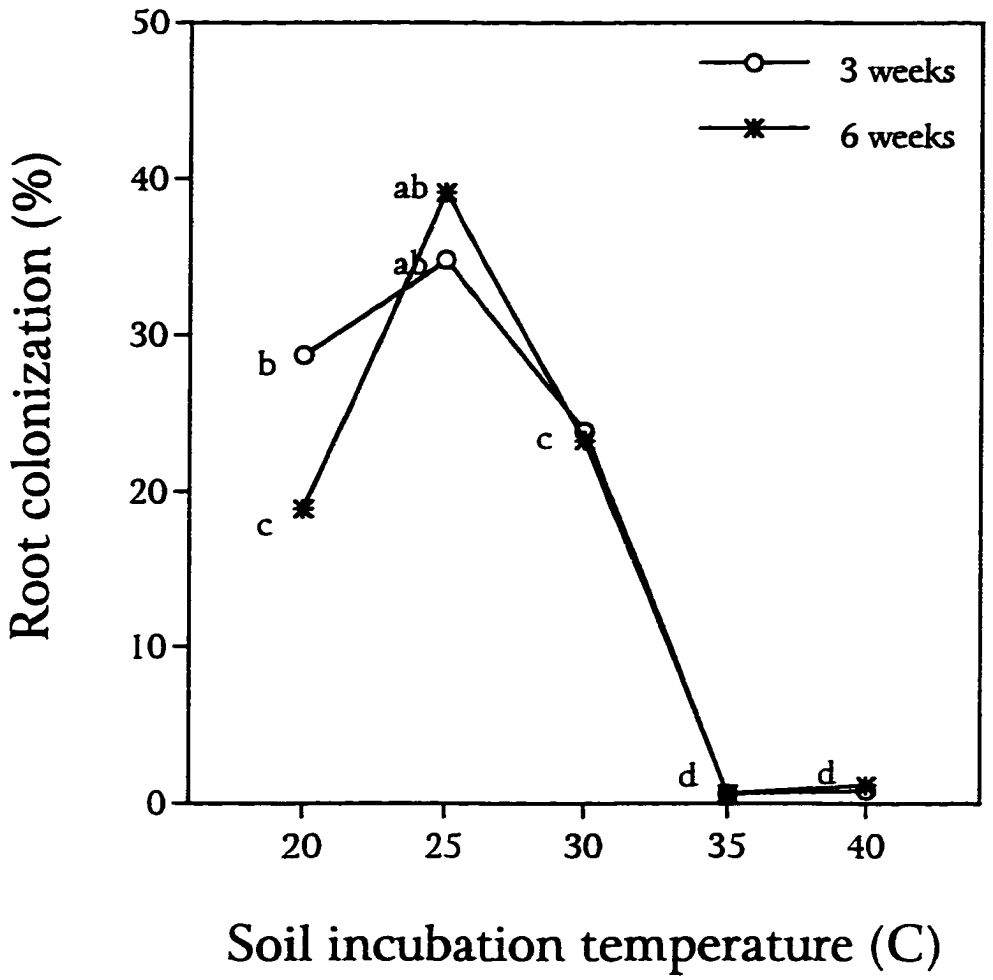

Fig.A2. Soybean root colonization by *Calonectria ilicicola* grown at 25°C for 8 weeks in a green house in soil infested with microsclerotia. The soils were previously incubated at 20, 25, 30, 35 or 40°C for 3, or 6 weeks. Treatment means marked with the same letter did not differ significantly according to least squares means ($P \leq 0.05$).

a negligible level of root colonization was detected in soybeans grown in infested soil incubated at 35 or 40^{0}C (Fig. A2).

Discussion

According to theses results optimal soil temperature range for microsclerotia germinability is between 20-30^{0}C and 35^{0}C is the high temperature limit. The number of germinable microsclerotia after incubating at different temperatures was apparently higher than that detected in experiments described in Chapter III. The age of the *C. ilicicola* cultures used to extract microsclerotia was 6 wks in those experiments, where as it was 8 wks in this experiment. Older cultures may have higher number of mature or older microsclerotia and these mature or older microsclerotia may withstand high soil temperature better than immature or younger microsclerotia. However, more information is needed before suggesting any relationship between temperature tolerance of *C. ilicicola* microsclerotia and their age. Microsclerotia exposed to 35 and 40^{0}C were not infective indicating that soil temperatures $\geq$ 35^{0}C have an lethal effect on these microsclerotia. Maximum infectivity was detected in microsclerotia exposed to 25^{0}C.

VITA

Pali Upulmathie De Silva Kuruppu was born in Hikkaduwa, Sri Lanka, and received her secondary education in Sri Sumangala College, Hikkaduwa, and Visaka College, Colombo, Sri Lanka. She received her bachelor of science degree in Botany from the University of Sri Lanka, in 1976. After working as an Instructor in the Department of Botany, University of Sri Lanka, for one year, she started her agricultural research career in the Department of Agriculture, Sri Lanka, in 1977. She was involved in plant pathological research in the same department from 1984 to 1994. She received her master of science degree in plant pathology from the University of Arizona, in 1989.

In Fall 1994, she joined the Department of Plant Pathology and Crop Physiology at the Louisiana State University to pursue her doctoral degree. She is a member of American Phytopathology Society, Gamma Sigma Delta The Honor Society of Agriculture and Sri Lanka Association for the Advancement of Science. She served as the Vice-President and President of the Graduate Student Association in the Department of Plant Pathology and Crop Physiology at L.S.U. She also served as the Public Relations and Publicity Associate Chairperson of the International Committee, Union Program Council, at L.S.U. She will receive the degree of Doctor of Philosophy at the December Commencement, 1998.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Pali Upulmathie De Silva Kuruppu

Major Field: Plant Health

Title of Dissertation: Factors Affecting Root Colonization by *Calonectria ilicicola* and Development of Red Crown Rot Disease on Soybean

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

(Co-chair)

Date of Examination:

July 24, 1998

IMAGE EVALUATION
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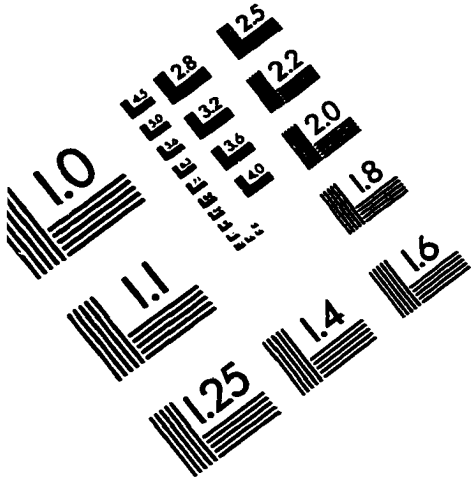

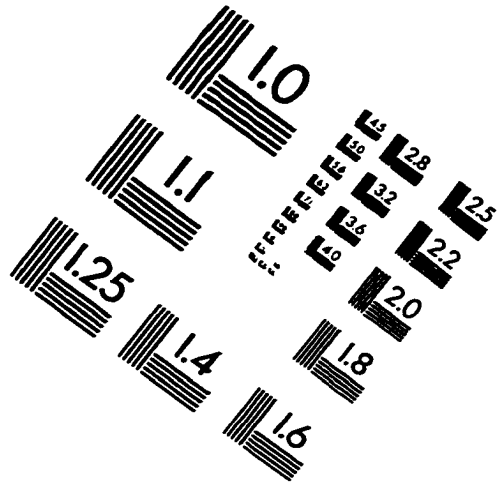

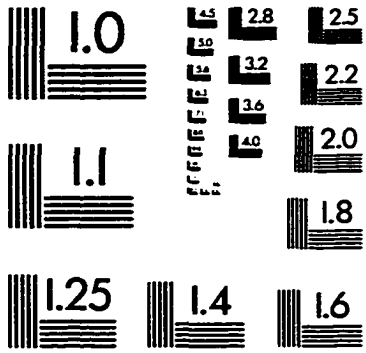

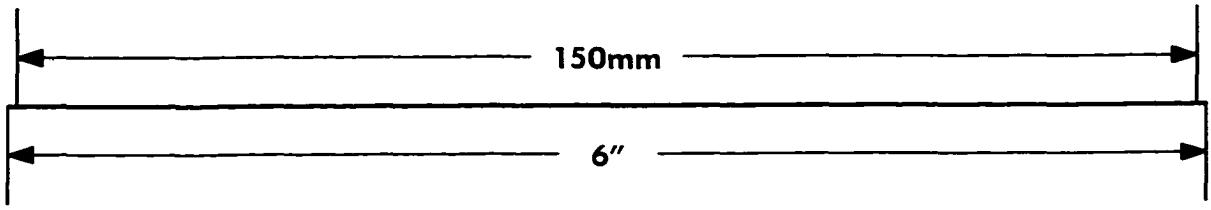

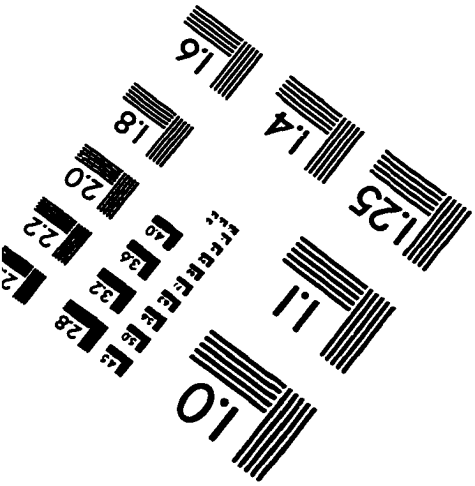

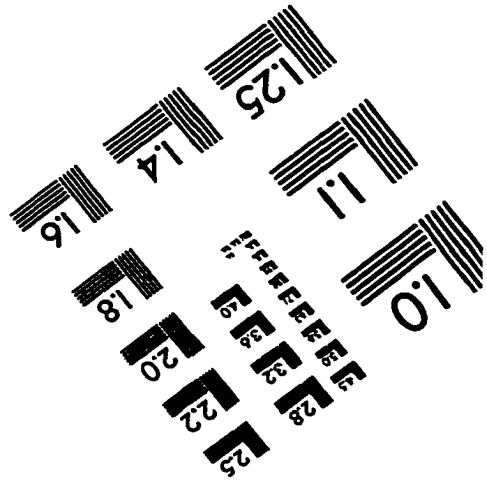

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